FUNCTIONAL CHARACTERIZATION OF DJ-1: AN OXIDATIVE RESPONSE PROTEIN

By
Casey M. Clements

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Approved by:

Advisor: Dr. Jenny P.-Y. Ting, Ph.D.

Dr. Blossom Damania, Ph.D.

Dr. Nancy Raab-Traub, Ph.D.

Dr. Lishan Su, Ph.D.

Dr. Yue Xiong, Ph.D.

ABSTRACT

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Casey M. Clements
(Under the direction of Jenny P.-Y. Ting)

The cancer and Parkinson's disease associated protein DJ-1 functions to protect cells from toxins. Presented here is a mechanistic analysis of DJ-1 cytoprotection. We show that DJ-1 is required for cellular responses to oxidant exposures leading to the protection and survival of cells in adverse conditions. We find that DJ-1 is required for the activity of Nrf2, the master regulator of antioxidant transcription. Furthermore, we show that DJ-1 provides this function by causing Nrf2 to dissociate from its cytsolic inhibitor protein, Keap1. This stabilizes Nrf2, preventing its ubiquitination and degradation in the absence of oxidative stress. DJ-1, therefore, maintains an active-ready pool of Nrf2 protein in cells to respond to oxidative stress.

We then present a survey of DJ-1 interacting proteins. We show that DJ-1 remains unbound when the protein is not oxidized, but that during periods of oxidative stress, cysteine-106 in DJ-1 oxidizes causing DJ-1 to bind other proteins. We

characterize the interaction of DJ-1 with Cezanne, a deubiquitinating enzyme and negative regulator of NF-κB. We show that DJ-1 is able to inhibit deubiquitinating enzymes *in vitro*, including Cezanne and that DJ-1 negates Cezanne mediated inhibition of NF-κB. Finally, we implicate a broader role of deubiquitinating enzymes in antioxidant responses showing that the ubiquitin editing protein, A20, inhibits the antioxidant transcription factor Nrf2.

The results presented herein provide evidence for a mechanism of DJ-1 function as a positive regulator of gene transcription during periods of oxidative stress. DJ-1 functions in this role to protect cells from cytotoxic exposures leading to increased cell survival. Loss of DJ-1 is realized in the death of oxidative sensitive cells, such as neurons in the case of Parkinson's disease. On the other hand, excess DJ-1 activity leads to inappropriate cytoprotection, survival, and cancer. The results summarized in this thesis identify the DJ-1/Nrf2 axis as a target of therapy for the treatment of both cancer and Parkinson's disease.

DEDICATION

This work is dedicated to my wife Lucinda without whom none of this would have been possible. She has been a constant source of inspiration, sympathy, and encouragement.

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LIST OF ABBREVIATIONS

(in alphabetical order)

ALS…….Amyotrophic lateral sclerosis

ARE……....Antioxidant response element

CRE…….cAMP response element

DMSO……..Dimethylsulfoxide

MEF……..Mouse embryonic fibroblast

NSCLC…….Non-small cell lung carcinoma

PD……..…….Parkinson's disease

siRNA……...……Small interfering ribonucleic acid

tBHQ……...….tert-Butylhydroquinone

TNFα.....Tumor necrosis factor alpha

CHAPTER I:

Introduction

INTRODUCTION

Mechanisms governing the balance between cell survival and cell death have remained major focuses of molecular and cellular biological research. These mechanisms play critical roles in the development of many important clinical problems. Cancer, heart disease, aging, and neurodegenerative diseases are all caused by disruption in the balance between cell survival and cell death. Major scientific advances in recent times have highlighted the importance of cell signaling cascades and apoptosis, an active mechanism governing cell death, in the cause of human diseases.

1.1 DJ-1 – a multifunctional protein regulating cell survival

Don Juan-1 (DJ-1) is a ubiquitously and abundantly expressed protein with a molecular weight of 24 kilodaltons (Nagakubo et al. 1997; Hod et al. 1999). Initially described for its ability to transform mouse 3T3 cells in culture (Nagakubo et al. 1997), DJ-1 plays an important role in governing cell survival. Unlike other proto-oncogenes, DJ-1 does not affect cell cycle progression or apoptosis in the classical sense. Instead, as the work of this thesis shows, DJ-1 governs the cellular response to environmental toxin exposure by activating transcriptional responses to oxidative stress. DJ-1 therefore, functions to protect cells from cytotoxic cell death. In the absence of DJ-1, oxidation sensitive tissues – such as neurons and spermatids, are differentially affected leading to their functional loss. When DJ-1 is over-expressed or over-active, cells are given a distinct survival advantage allowing them to survive in environments that are normally

toxic, leading to prolonged mutagenic conditions eventually culminating in the development of cancers.

1.2 DJ-1 and cancer

Initially described for its ability to transform mouse fibroblasts, DJ-1 is a proto-oncogene of previously unclear function that is weakly carcinogenic in cell culture on its own, but cooperates in transformation with the oncogene, H-Ras (Nagakubo et al. 1997). The combination of DJ-1 and H-Ras was three fold more efficient at transforming 3T3 cells as was the Ras/Myc combination, although DJ-1 alone is able to transform cells with similar potency as the c-Myc oncogene. While this transforming capability is striking, the clinical significance of DJ-1 with respect to cancer remains unclear. However, several lines of evidence point to DJ-1 as a potentially important biomarker. First of all, DJ-1 has been shown, by our group and others, to be expressed at higher levels in tumor tissue than in normal tissue of the same type (MacKeigan et al. 2003; Hod 2004; Kim et al. 2005). Secondly, high expression of DJ-1 in non-small cell lung carcinoma cancers has been shown to be prognostic, directly correlating with patients' time to relapse (Kim et al. 2005). Lastly, DJ-1 can be directly quantified both from tissue samples, and in the blood (Le Naour et al. 2001; Allard et al. 2005; Melle et al. 2007). While it is unclear if DJ-1 is actively secreted into the blood and interstitial fluid, or if DJ-1 is spilled from damaged cells or tissues, non-invasive clinical screening of blood fluids for cancer biomarkers is promising.

1.3 DJ-1 and Parkinson's disease

Parkinson's disease is a neurodegenerative disorder affecting motor neuron control of movement. Parkinson's disease is very common, with an incidence and prevalence that increases proportionally with aging, affecting roughly 1% of persons over 65 years of age worldwide (Dawson et al. 2003). The overwhelming majority of Parkinson's disease cases are idiopathic, without an identifiable cause and despite intense research, the etiology of Parkinson's disease remains unclear. The diagnosis of Parkinson's disease is a clinical diagnosis based on a set of symptoms including, resting tremor, 'ratcheting' rigidity, bradykinesia, and postural instability (Tuite et al. 2007). Experienced clinicians can diagnose Parkinson's disease in seconds by observing these symptoms, having a patient walk a few steps, and by conducting a neurological physical examination. While the primary deficit in Parkinson's disease patients is their motor control, Parkinson's disease can also affect other neurological pathways as well. These effects are not present uniformly in Parkinson's disease patient populations, and their association with Parkinson's disease pathology is unclear. Such symptoms include cognitive, behavioral, and autonomic neurological dysfunction. (reviewed in(Zesiewicz et al. 2006)).

There are two histological hallmarks of Parkinson's disease. The first hallmark of Parkinson's disease is the progressive loss of neurons that make dopamine (dopaminergic neurons), specifically those found within the substantia nigra pars compacta of the human brain (Hodaie et al. 2007). The substantia nigra lies within the basal ganglia of the brain, and dopaminergic neurons act as an inhibitory signal extending to the caudate and putamen which coordinate motor neurons in the cerebral cortex. Figure 1.1 is a simple

schematic of the neuronal connections controlling movement as it relates to Parkinson's disease. Loss of dopaminergic neurons within the substantia nigra, as in Parkinson's disease, leads to hyper excitation and over activity of the cortex motor neurons producing the ridigity, tremor, and other physical symptoms and signs of Parkinson's disease (Hodaie et al. 2007). The second histological hallmark of Parkinson's disease are Lewy bodies, which are cytoplasmic inclusions within neurons. Lewy bodies are large insoluble aggregates of lipid and protein(Pollanen et al. 1993), largely comprised of α-synuclein(Lippa et al. 1998), Lewy bodies also containing ubiquitin(Lowe et al. 1988) and highly oxidized proteins (Good et al. 1998; Giasson et al. 2000). While dopaminergic neuronal loss is characteristic of Parkinson's disease, Lewy bodies, most commonly found post-mortem in Parkinson's disease patients, are also associated with related neurodegenerative disorders, most notably Dementia with Lewy Bodies, a syndrome resembling Alzheimer's disease (Gibb et al. 1987).

While there is no cure for Parkinson's disease, several treatments are used to alleviate the motor symptoms of the disease. Currently, the overwhelming majority of medical and surgical treatment modalities for Parkinson's disease are aimed at potentiating dopamine signals. Pharmacologic preparations of L-Dopa known as levodopa, are orally available and can cross the blood-brain barrier where they are metabolized to dopamine, globally elevating dopamine levels within the brain. Peripheral metabolism of the L-Dopa is not insignificant, and combination therapies are available containing inhibitors of peripheral dopamine metabolism thereby increasing the dopamine delivery to the brain. Similarly, within the brain, dopamine is inactivated by enzymes including Catechol-O-methyl transferase (commonly known as COMT), and

Monoamine oxidase-B (MAO-B). (reviewed in(Napolitano et al. 1995)) Inhibitors of these enzymes prolong dopamine half-life allowing dopamine to remain active within synapses for longer periods of time producing a more robust, potentiated dopamine signal. (reviewed in(Siderowf et al. 1999)) Surgical techniques are also available to help treat Parkinson's disease symptoms, and are used in severe cases. These include deep brain stimulation, in which an electronic device is implanted into subthalamic nuclei or the globus pallidus, where the device delivers high frequency electric pulses to the surrounding tissue. It remains unclear how deep brain stimulation specifically inhibits surrounding neural pathways, but the procedure often improves patients symptoms, and it is being used with increased frequency (Wichmann et al. 2006). More drastically, surgery can remove or destroy either the subthalamic nucleus or the globus pallidus sections of the brain. These structures contain neurons that inhibit the dopaminergic neurons of the substantia nigra. Therefore, ablation of these inhibitory neurons leads to increased dopamine neurotransmission, and decreased motor neuron excitation – reducing the symptoms of Parkinson's disease.

In a groundbreaking study published in January 2003, Bonifati et. al. described two consanguous familes in Europe that lacked DJ-1 expression, and had early onset Parkinson's disease (Bonifati et al. 2003). Patients that were homozygous for DJ-1 loss contracted the disease with a genetic penetrance of 100%. One of these two families, the Dutch kindred, carried a large genomic deletion of DJ-1, spanning exons 1-5 of the PARK7 gene that encodes for DJ-1, leading to no expression of the DJ-1 protein. The other family, an Italian kindred contained a point mutation within the DJ-1 open reading frame resulting in a single amino acid substitution at residue 166 from a leucine in the

wild type, to a proline in the mutant. Subsequent molecular analysis of this mutant has shown that the mutation leads to misfolding of the DJ-1 protein destabilizing it and leading to the degradation of the mutant protein product (Miller et al. 2003). Therefore the point mutation, like the genomic deletion, also results in a DJ-1 null phenotype amounting to the same effect, early onset Parkinson's disease. Following Bonifati et. al., several other groups have identified mutations in DJ-1 within early onset Parkinson's disease patients, these are reviewed in Lev et. al., 2006 (Lev et al. 2006).

DJ-1 deficient Parkinsonism has since been clinically characterized in several small scale clinical studies (Abou-Sleiman et al. 2003; Dekker et al. 2003; Ibanez et al. 2003; Healy et al. 2004; Hedrich et al. 2004; Tan et al. 2004; Klein et al. 2005). True DJ-1 deficient Parkinson's disease is very rare, accounting for 1-2% of early onset Parkinson's disease patients, a very small subset of idiopathic Parkinson's disease, therefore making up only a minute portion of Parkinson's disease cases as a whole. However, other studies have identified genetic polymorphisms in the park7/dj-1 gene in normal incident Parkinson's disease that may play a role in Parkinson's disease development or progression that have not yet been studied at length (Eerola et al. 2003).

Parkinson's disease resulting from the loss of DJ-1 is virtually indistinguishable from most other cases of Parkinson's disease arising in the general population apart from the early onset of the disease (Dekker et al. 2004; Schweitzer et al. 2007). The disease course is diagnosed at a relatively young age for Parkinson's disease, ranging from 27 to 40 years of age at onset. The clinical progression of the disease is slow, on the order of years, and patients have been successfully treated using standard dopamine replacement therapies including Levodopa (Abou-Sleiman et al. 2004).

While Parkinson's disease caused by DJ-1 loss may in fact be a rare occurrence, the causative association of DJ-1 with Parkinson's disease is of particular interest since the underlying etiology of Parkinson's disease has remained elusive despite intense research spanning the last several decades. In this vein, the intent of research relating to DJ-1 function, is that in understanding how DJ-1 functions at a cellular or molecular level will allow an understanding of how the loss of those functions could lead to Parkinson's disease. Subsequently this research aims to define how dysfunction of these and related mechanisms may relate to the causes of Parkinson's disease as a whole, with the eventual goal of identifying targets of therapy treating the root causes of Parkinsonism to prevent or eliminate Parkinson's disease as a whole. Rare genetic causes of Parkinson's disease or Amyotrophic Lateral Sclerosis, which is also associated with monogenetic causes, arguably present the best opportunities to study the causes of neurodegenerative diseases, none of which have ever been cured.

1.4 DJ-1's role in other neurodegenerative diseases

While the strongest disease association with DJ-1 remains Parkinson's disease, alterations in normal DJ-1 sequence or expression have been observed in several other neurodegenerative diseases. While the clinical or biological significance of these observations remain unclear, taken together they portray a wider functional role for DJ-1 in cell survival, particularly with respect to brain tissue. Immunodetection of DJ-1 has failed to place DJ-1 in Lewy body protein inclusions found in Parkinson's disease or in Dementia with Lewy Bodies, however, DJ-1 has been identified as a component of other protein inclusion bodies. DJ-1 has been shown to be present in Tau lesions (Rizzu et al.

2004) within the brain that are associated with dementia syndromes, most notably Alzheimer's disease and Pick's disease. Similarly, DJ-1 along with α-synuclein have been identified within astrocyte cytoplasmic inclusion bodies found in patients suffering from multisystem atrophy (as opposed to α-synuclein inclusion bodies in neurons, namely Lewy bodies) (Neumann et al. 2004).

Both the Dutch and Italian families initially used to associate DJ-1 with Parkinson's disease also suffer psychiatric symptoms of unknown significance. These families carry very different mutations, both leading to a DJ-1 null phenotype; yet both families have a history of behavioral and dystonic disturbances along with severe anxiety and some psychotic episodes (Bonifati et al. 2003). These two families provide a case study on DJ-1 and neural dysfunction, but are too few in number to adequately associate DJ-1 with psychiatric disorders at large. No further studies have addressed this question to date, but the finding is intriguing. In 2005, a different Italian family was identified carrying a DJ-1 mutation, suffering from multiple neurodegenerative disorders including Parkinson's disease, amyotrophic lateral sclerosis (ALS), and dementia (Annesi et al. 2005). Recent research has found many overlapping mechanisms between neurodegenerative diseases. A good example includes the role of oxidative stress in Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. The association of this second Italian family with DJ-1 mutation further indicates a broad role for DJ-1 function in the context of neuronal protection.

1.5 Structural biology of DJ-1 protein

Following the clinical correlation of DJ-1 with Parkinson's disease, several groups rushed to crystallize DJ-1 protein, hoping to determine the indispensable role that DJ-1 plays. In the summer of 2003, five independent groups published reports of DJ-1 crystal structures (Honbou et al. 2003; Huai et al. 2003; Lee et al. 2003; Tao et al. 2003; Wilson et al. 2003). However, these reports varied significantly in their interpretation of the resolved structure.

DJ-1 belongs to an expanding protein family known as the DJ-1/PfpI/ThiJ protein family with representative orthologs spanning across evolution from archaebacteria to eukarotic species (Tao et al. 2003). This conservation of protein sequence and structure suggests that DJ-1 family proteins provide a vital function to living cells. The functions of the DJ-1 family member proteins however, are diverse and various. Proteins in this family include: PfpI and PH1704 – bacterial cysteine proteases (Halio et al. 1996; Du et al. 2000), ThiJ – a glutamine aminotransferase and kinase (Mizote et al. 1999), HPII – a bacterial catalase (Loewen et al. 1993), and Hsp31 – a protein chaperone and heatshock protein in E. Coli (Sastry et al. 2002). Obviously, unifying the common structure of these proteins with the disparate molecular functions is a daunting task.

The resolved structure of DJ-1 reveals a helix-strand-helix sandwich configuration consisting of eleven beta sheets, and eight alpha helices. Several of the reports compare DJ-1 protein structure to other members of the protein family that have previously been crystallized and published (Honbou et al. 2003; Huai et al. 2003; Lee et al. 2003; Tao et al. 2003; Wilson et al. 2003). Structurally, DJ-1 matches up remarkably well with other proteins in the family, with the exception of DJ-1 having an extra alpha

helix at the carboxy terminus of the protein. This extra helix is suggested to have important consequences on any enzymatic function DJ-1 might possess since it blocks a deep groove in the DJ-1 protein that is analogous to the active site in other protein family members. One report suggested that this extra helix may regulate potential enzymatic activity of DJ-1 possibly in response to oxidation, but no experimental evidence has been shown to support this hypothesis to date (Honbou et al. 2003). This extra helix is additionally involved in an interface between two DJ-1 molecules, forming a homodimer. All five reports found that DJ-1 crystallized as a homodimer, and Wilson et al.verified that DJ-1 also forms dimers in solution using native gel electrophoresis and gel filtration chromatography (Wilson et al. 2003). Subsequent studies have shown that DJ-1 does self-associate in cells (Gorner et al. 2007; Hulleman et al. 2007). Therefore it is likely that DJ-1 dimerization is important for DJ-1 function.

Along with wild type DJ-1, several DJ-1 mutants were crystallized over the course of these studies as well. Mutation of the site of DJ-1 sumoylation, a post-translational modification that regulates the cellular localization and activity of many proteins, Lys130, to an arginine did not affect DJ-1 structure significantly (Tao et al. 2003). On the other hand, mutation of Leu166 to a proline, one of the causative mutations in DJ-1 linked Parkinson's disease, severely disrupted one of the alpha helices in DJ-1, preventing DJ-1 dimerization (Tao et al. 2003). This leads to protein destabilization and rapid degradation in the cell, a phenomenon that has been shown by several groups (Miller et al. 2003; Olzmann et al. 2004). Also of note, Lee et. al. crystallized DJ-1 protein that was oxidized by pretreatment with hydrogen peroxide (Lee et al. 2003). Hydrogen peroxide treatment of DJ-1 altered the electron density of cysteine

106, a fact that was earlier suggested given that Cys106 was sensitive to radiation induced damage during X-ray diffraction (Wilson et al. 2003). Measurements of the oxidized protein suggest that the cysteine at 106 is likely oxidized to a sulfinic acid to fit the crystal measurements (Lee et al. 2003).

While the various activities of DJ-1 family members are diverse, many of these proteins are in fact enzymes. DJ-1 protein family members containing enzymatic activity catalyze reactions by using a reactive cysteine residue to facilitate proton transfer between nearby amino acid residues to hydrolyze substrates. This process classically uses a catalytic triad of amino acids in close proximity to one another and arranged in a particular order. DJ-1 family member proteins are no different; however, when crystal structures of DJ-1 were resolved, only the cysteine (residue 106 in DJ-1) was conserved between DJ-1 and the catalytic triad present in other DJ-1 family members. Protein folding contributes a possible replacement for another of the classical triad, a histidine (residue 126 in DJ-1), but this particular histidine is not well conserved among other DJ-1 family members suggesting that if it does contribute to an enzyme active site with DJ-1, this may be an emergent property of DJ-1 not present in members of the protein family. Finally, while other members of the protein family have an acidic aspartic or glutamic acid residue within their enzymatic active site, no such residue exists in DJ-1. It was proposed that a glutamic acid at position 18 in DJ-1, or an aspartic acid at the 24th position in adjacent dimerized DJ-1 molecules could contribute to a DJ-1 active site (Honbou et al. 2003; Honbou et al. 2003), but subsequent crystal measurements have shown this to be unlikely (Huai et al. 2003; Tao et al. 2003; Wilson et al. 2003). An unconventional active site in the DJ-1 protein cannot be ruled out. The Cys106, and

His126 residues are in the correct orientation to act as a catalytic diad, which are found in other important protein families including caspases.

Despite the lack of a classical catalytic triad in DJ-1, several of these studies have tested for DJ-1 enzymatic activity (Huai et al. 2003; Lee et al. 2003; Tao et al. 2003; Wilson et al. 2003). Unlike similar protein family members, DJ-1 has no protease activity against a slew of potential substrates, including unbiased short peptides. Neither was it able to act as an aminotransferase or a kinase targeting thiamine or pyridoxine derivatives, as some DJ-1 family members can. The lack of a proposed enzymatic function of DJ-1 lead Wilson et. al. to suggest that DJ-1 is likely to play a role in gene expression or as a molecular chaperone (Wilson et al. 2003). Indeed, Lee et. al. tested DJ-1, activity in preventing protein aggregation of traditional chaperone substrates, Luciferase and the protein 'CS'. DJ-1 was able to act as a chaperone for both of these proteins *in vitro* (Lee et al. 2003).

1.6 Animal models of DJ-1 function

Several orthologs of DJ-1 have been cloned. Soon after human DJ-1 was identified, the rat DJ-1 ortholog was cloned as well. Designated 'Contraceptive Associated Protein-1' (CAP-1), the rat DJ-1 ortholog was published to be indispensable for male fertility in rats (Wagenfeld et al. 1998). While this result has not been duplicated to date, mice genetically ablated for DJ-1 are able to reproduce normally (Goldberg et al. 2005; Kim et al. 2005), as are human beings that carry genetic deletion of both DJ-1 loci, or DJ-1 null mutations (Bonifati et al. 2003). In fact, DJ-1 has been highly conserved through out the course of evolution. One study in particular is striking,

in which the sequences of all DJ-1 family proteins from mammals ranging back to prokaryotes were compared using traditional methods of sequence analysis. The researchers came to the conclusion that DJ-1 family members were very closely related, and that the researchers could not adequately be distinguish between eukaryotic species by protein sequence alone (Wei et al. 2007). This suggests that DJ-1 function is important and has been maintained throughout recent biological history. However, one exception to this rule is drosophila DJ-1.

1.7 Drosophila models of DJ-1 function

In *Drosophila Melanogaster*, two separate DJ-1 genes encoding separate protein products have arisen over the course of evolution. Named DJ-1α and DJ-1β, the drosophila orthologs have different expression patterns, yet by protein sequence remain very similar to one another and to DJ-1 in higher order species (Menzies et al. 2005). DJ-1β has a similar expression pattern as human DJ-1, expressed globally throughout the fly, at varying levels based on cell type. DJ-1α on the other hand has limited expressed, most notably in the testes of the fly, and at very low levels in other tissues including the brain. Several molecular models have been used to ascertain the function of drosophila DJ-1. These models all use techniques that reduce or eliminate DJ-1 expression in the fly, either globally or in a tissue specific manner. The authors of these studies then observed the flies for resulting gross phenotypes, and changes in cellular biology.

Three independent groups have used P-element transposition and microdeletion or insertional inactivation of the DJ-1 locus to genetically alter the genes encoding DJ-1 in the drosophila genome yielding DJ-1 knockout flies (Menzies et al. 2005; Meulener et al.

2005; Park et al. 2005). Two of these groups targeted the DJ-1β locus, justifying their choice due to DJ-1β's similarity in expression to human DJ-1, and the apparent lack of substantial levels of DJ-1α in the drosophila brain. In both cases the resulting DJ-1 deficient flies did not display the characteristic histological hallmark of Parkinson's disease, loss of dopamine expressing neurons (Menzies et al. 2005; Park et al. 2005). Neither did the flies have a frank Parkinson's disease like phenotype of motor deficits, though one of the groups did observe a decreased climbing ability of the flies that progressed with age. However, this phenotype was apparent even in one-day-old flies, and no transgenic experiment was performed adding DJ-1β expression back to these flies to determine the specificity of the observed phenotype (Park et al. 2005). This draws into question both the role that DJ-1 plays in this phenotype as opposed to the role of the genetic background of the DJ-1 versus control fly strains, as well as the significance of the perceived motor deficit in these flies as it could relate to human Parkinsonian degeneration.

When the DJ-1β locus was inactivated by Menzies et. al., the flies were, to a large extent, normal. However, at sixty days of age the researchers observed increased survival of dopaminergic neurons in the drosophila brain (Menzies et al. 2005). This phenotype is exactly opposite of what is seen in Parkinson's disease, and in functional analyses using mammalian DJ-1. At first glance this finding seems to be in stark contrast to every published report of DJ-1 function, in which DJ-1 loss is associated with increased cell death. However, the researchers went on to show that following DJ-1β gene inactivation,

DJ-1α expression is coordinately upregulated in the brains of these flies by a factor of two fold, perhaps leading to cytoprotection. This could imply that the DJ-1α protein plays a substantial role in specific dopaminergic neuronal protection in drosophila, since serotonergic neurons in the same areas of the brain were unaffected by DJ-1 alterations in this model.

Meulener et. al. used P-transposition genetic deletion to generate double knockout flies deficient in both DJ-1α and DJ-1β gene loci (Meulener et al. 2005). This strain of drosophila did not show any motor deficits, and numbers of dopaminergic neurons in the brains of these flies were unaffected by DJ-1 ablation. However, the timing of these experiments may have missed important differences since the researchers chose to study the flies at thirty days of age at the oldest. The authors report instead another important finding using this model. DJ-1 deficient flies were selectively sensitive to treatment with oxidant chemicals including rotenone, paraquat, or hydrogen peroxide. DJ-1 double knockout flies were killed at only one-tenth the dose of paraquat needed to kill wild type drosophila, and one-fifteenth the dose of hydrogen peroxide (Meulener et al. 2005). Strikingly, the dopaminergic neurons in the brains of these flies were unaffected in these oxidant treatments, indicating that these cells were not selectively sensitive in this model. This could indicate that DJ-1 may play a role more broadly cell survival throughout the body, a finding that is clinically superceded by the Parkinson's disease association with DJ-1 loss, but perhaps still a significant physiological role of DJ-1.

As an alternative to genetic ablation of DJ-1 genes within the fly germline, Yang and colleagues used RNA interference (RNAi) to knockdown DJ-1 expression at the

mRNA level reducing DJ-1 protein expression (Yang et al. 2005). This group chose to target DJ-1α specifically. They justify this choice since DJ-1α contains the same three amino acid residues that have been proposed by structural analysis to form an enzymatic catalytic triad, although no functional data has shown enzymatic activity of DJ-1 protein to date. DJ-1β on the other hand has one of those three amino acids altered compared to human DJ-1 protein. RNAi knockdown of DJ-1α did not affect DJ-1β protein levels even though the coding sequence of both genes is very similar. When DJ-1α RNAi was expressed throughout the developing fly body, the resulting strain was lethal at the larval stage. By combining placement of the DJ-1 RNAi under control of a GAL4 responsive promoter sequence, and tissue specific expression of GAL4, the group was able to express the DJ-1 RNAi specifically within the tissue of the drosophila eye. This induced a 'rough' eye phenotype that was characterized by loss of photoreceptor neurons within the eye. Furthermore, this phenotype was dose dependent on the expression of the DJ-1α RNAi, with high RNAi expression and therefore low DJ-1α expression leading to a more striking degeneration of the eye. These findings can be criticized due to the use of only one DJ-1 specific RNAi sequence and the lack of control non-targeting RNAi sequences, important add-back experiments somewhat reduce the risk that their findings are due to off target effects of the DJ-1α RNAi. When DJ-1α RNAi is expressed at high levels leading to severe eye degeneration, restoring the expression of DJ-1α or human DJ-1 is able to partially rescue the eye phenotype. This provides the first evidence that DJ-1 function could be conserved between drosophila and mammals. The authors went on to test the role of DJ-1α in dopaminergic pathways within the drosophila brain. Loss of

DJ-1α expression in neurons cultured from drosophila brains were markedly sensitive to oxidative cell killing. This was true when the cultures were treated with either exogenous hydrogen peroxide or inhibitors of catalase that mimic endogenous oxidative stress. While DJ-1α was shown to be able to scavenge hydrogen peroxide to a small extent itself, it is two orders of magnitude less efficient than catalase, suggesting that its role as a scavenger is unable to account for the observed robust cytoprotective phenotype. It should be noted here that previous publications on drosophila DJ-1α were unable to show that DJ-1α overexpression was protective of oxidant cell killing (Menzies et al. 2005). These findings may not be at odds with one another if DJ-1α is not the limiting factor in these cytoprotective pathways; in which case, DJ-1α loss may evidence an important role, while over-expression may have little effect.

Expression of DJ-1α RNAi using neuronal specific GAL4 expression led to an age dependent loss of dopaminergic neurons in the dorsomedial cluster, an area of the drosophila brain often studied as a model of Parkinson's disease. Additionally, the overall content of dopamine in the brain was decreased in the absence of DJ-1α expression. It has been suggested that the method used by Yang et. al. to determine dopamine neuronal degeneration may be outdated and unable to provide data sufficient to come to the conclusions that DJ-1α loss leads to dopaminergic neuron degeneration (Moore et al. 2006). Furthermore, the authors did not sufficiently study the effects of DJ-1α on other neurons in drosophila. While these results are very promising, and indicate that DJ-1α is likely very important in drosophila cell survival, further studies are

necessary to show the specificity of DJ-1α in dopaminergic neuronal cytoprotection in the drosophila brain, and how this may act as a model for DJ-1 deficient Parkinson's disease.

The transgenic RNAi mediated method of DJ-1α knockdown was used to determine possible genetic interactions of DJ-1α with signaling pathways in drosophila (Yang et al. 2005). Using the dose dependence of the DJ-1α protein knockdown with the eye degeneration phenotype, they examined signaling pathway mutants that could exacerbate the mild effect of low DJ-1α RNAi expression or abrogate the effects of extreme DJ-1α loss. While no genetic association of DJ-1α was seen with either the Ras1/MAPK or JNK signaling pathways, the authors observed a strong association or DJ-1α with the PI3K/Akt pathway. This is based on the observation that mild 'rough' eye phenotypes from low DJ-1α RNAi expression were drastically augmented when either PTEN, a negative regulator of PI3K signaling, or a dominant negative form of PI3K was expressed along with low DJ-1α RNAi. This despite the fact that dominant negative PI3K had little effect on eye phenotype in the absence of DJ-1α knockdown, or in a different model of eye degeneration using the *tau* gene. Furthermore, the severe eye phenotype from high DJ-1α RNAi expression was mitigated by coexpression with supra-physiologic PI3K. This association is bolstered by evidence showing that while Akt levels in the brains of wild type and DJ-1α knockdown flies are similar, Akt is phosphorylated to a lesser extent in the absence of DJ-1α, suggesting that the PI3K signaling pathway is damaged in the absence of DJ-1α in drosophila.

1.8 Mouse models of DJ-1 function

Similar to drosophila, researchers have used mice as genetic models of human gene function for many years. While technically more difficult, genetic engineering of mice provides a mammalian model that more closely relates to human beings, and therefore is usually more physiologically relevant. Following the disease association of DJ-1 malfunction with Parkinson's disease, several research groups generated mouse models of DJ-1 function. Researchers from diverse scientific backgrounds have used mouse models to provided divergent opinions on DJ-1 function with data generated from different systems.

Martinat et. al., published the first report of mouse DJ-1 function from primary non-transfmored cells (Martinat et al. 2004). They generated murine embryonic stem (ES) cells lacking DJ-1 expression. These cells were created by taking advantage of a previously existing ES cell line that harbored a retroviral insertion with the DJ-1 gene that disrupts a splice site within the DJ-1 transcript. The mutant transcript leads to a truncated form of DJ-1 lacking the carboxy terminus of the normal protein, leading to destabilization of the mutant, degradation, and a DJ-1 null phenotype. ES cells generated in this way are sensitive to cell killing by oxidative stress via hydrogen peroxide or copper treatment, and proteosomal inhibition by the chemical lactacystin. ES cells lacking DJ-1 were not any more sensitive to cell death induced by endoplasmic reticulum stress from tunicamycin, global kinase inhibition by staurosporine, or from protein translation inhibition by cycloheximide. These findings suggest that mouse DJ-1 has specificity for cytoprotection against oxidative stress. The authors went on to further

describe the sensitivity of DJ-1 null ES cells by inducing oxidative stress, and measuring the oxidation state inside of the ES cells. They measured the amount of reactive oxygen species directly, and as a marker of oxidative cell damage they measured the carbonylation of cellular proteins. Cells lacking DJ-1 expression exhibited the same levels of reactive oxygen within the cells compared to wild type ES cells, and similarly, protein carbonylation one hour following oxidative treatment was indistinguishable. However, six hours after oxidative burst, the protein carbonylation measurement of oxidative cell damage was much higher in the DJ-1 null cells than in wild type ES cells. This evidence indicates that DJ-1 is involved in the response to oxidative stress.

Using established methods, Martinat et. al. differentiated their ES cells into dopaminergic neurons in culture (Martinat et al. 2004). ES cells lacking DJ-1 produced far fewer dopaminergic neurons when differentiated than wild type expressing ES cell clones. This effect of DJ-1 seems to be specific since other neuronal cell types were unaffected when generated from DJ-1 null cells. Therefore, to further test the specificity of DJ-1 function in dopaminergic neurons, differentiated cultures were treated with 6-hydroxydopamine, an oxidant compound which can serve as a substrate for the dopamine transporter, and is therefore specifically concentrated inside of dopaminergic neurons. DJ-1 null neuronal cultures were more sensitive than wild type cultures to 6-hydroxydopamine cell killing. As a correlate to these findings, Martinat et. al. used lentiviral delivery of RNAi to knockdown DJ-1 expression in the developing midbrain of normal wild type mice. Dopaminergic neurons in slices from these brains were specifically sensitive to cell death from hydrogen peroxide, while gabaergic neurons, which make up most of the cell bodies within the developing mouse midbrain, were

unaffected. The authors suggest that DJ-1 has a specific effect on dopaminergic neuron survival in response to oxidative stress. It remains unclear however, what role the general senstivity of dopaminergic neurons to oxidative cell killing (vs. other neuronal types) contributes to this effect as opposed to their model of DJ-1 specificity of function within said neurons.

Goldberg et al. generated the first live mouse lacking DJ-1 expression (Goldberg et al. 2005). This DJ-1 knockout strain was generated by homologous recombination to remove the second exon of the DJ-1 gene. This removes the translation start codon within the open reading frame, while not affecting transcription of the mutant DJ-1 transcript. The authors acknowledge that in frame start codons in the third, fifth, sixth, and seventh exons could lead to the translation of truncated forms of DJ-1, but argue that since polyclonal antibodies raised against full length DJ-1 do not detect smaller forms of mutant DJ-1 protein, this risk is minimal.

Somewhat surprisingly, these DJ-1 knockout mice appear normal, and the authors provide several pieces of negative data on unchanged phenotypes between these mice and their wild type counterparts. The Goldberg et. al. knockout mice did not contain Lewy bodies, produced the same amount of dopamine, metabolized dopamine similarly, and had no difference in the number of dopaminergic neurons at either 3 months or 12 months of age. Furthermore, oxidative stress generated by paraquat treatment did not adversely affect DJ-1 knockout dopamine neurons any more than those expressing normal levels of DJ-1 in vivo. However, it may be noteworthy that the authors only report using a single paraquat dosage schedule, which may not be adequate to tease out differential sensitivity. The authors then measured evoked dopamine overflow within

brain slices from these knockout mice. This method uses electrophysiology to measure the effect of dopamine on a tissue sample when a dopaminergic neuron is stimulated to release its stored neurotransmitter. Evoked dopamine overflow from knockout mouse brain slices was only one sixth of that from wild type controls. Dopamine overflow can be altered due to differences in dopamine release from neurons, or reuptake removing dopamine from the extracellular milieu. Treatment with the dopamine transporter inhibitor nomifensine eliminated the difference in dopamine overflow, indicating that the difference in this case is due to reuptake by the dopamine transporter. Dopamine transporter activity was increased in DJ-1 knockout brains independent of transporter expression, which is unaltered as measured by quantitative PCR and radioligand binding.

Treatment of brain slices with high dose dopamine causes dopaminergic neurons to hyperpolarize, preventing firing. This membrane hyperpolarization fades over time, and firing can resume (Goldberg et al. 2005). Given the effect of DJ-1 on dopamine overflow, Goldberg et. al., tested the effect of DJ-1 on this dopamine specific function. Brain slices from DJ-1 knockout and wild type control mice were treated with dopamine, and mice lacking DJ-1 interrupted firing following dopamine treatment for a much shorter period of time than wild type animals. This presynaptic inhibitory effect of dopamine is a known effect of the dopamine 'D2' receptor (Lacey et al. 1987; Mercuri et al. 1997). In agreement with that model, treatment of the brain slices with the D2 specific agonist, quinpirole, produced a robust inhibition of firing in wild type mice, but that effect was reduced by half in mice lacking DJ-1. This led the authors to further study the effects of DJ-1 on the dopamine D2 receptor, and determine specificity of DJ-1 in effecting that receptor. In mice, projections from the dopaminergic neurons in the

substantia nigra pars compacta extend to the innervate medium spiny neurons in the striatum. Dopamine mediates the effects of long term potentiation (LTP), and long term depression (LTD), in these synapses to regulate synaptic plasticity thought to be involved in motor learning (Calabresi et al. 2000). Both LTP and LTD are effects of a high frequency of firing in the presynaptic neuron, modifying subsequent signals either strengthening them or weakening them respectively. These effects are receptor specific. While both the dopamine D1 and D2 receptors are responsible for LTP, it is specifically the dopamine D2 receptor responsible for LTD (Thomas et al. 2000). While DJ-1 knockout mice had normal presynaptic neuron firing and synaptic transmission, mice lacking DJ-1were unable to evoke an LTD while their responses to LTP remained intact (Goldberg et al. 2005). Exogenous addition of the D2 specific receptor agonist restored LTD, while the D1 agonist, SKF38393, did not.

In agreement with these findings of motor neuron dysfunction in the absence of frank dopaminergic neuronal cell death, these DJ-1 knockout mice do not develop a Parkinson's disease phenotype, but instead are hypokinetic. When measured in an open field, DJ-1 knockout mice move less than their wild type counterparts, and spend less time "reared" up on their hind limbs. However, unlike these voluntary movements, reflexes, which do not depend on the activity of the motor cortex, were unaffected in DJ-1 knockout mice.

Similar to Goldberg et. al., Pisani et. al., also approached the function of DJ-1 from the perspective of neurobiology (Pisani et al. 2006). In collaboration with the Shen lab, this group used the same mouse model described above. Parallel to the inhibition of firing following dopamine treatment, this group examined hyperpolarization following

oxygen and glucose deprivation. Unlike with dopamine treatment, they find that DJ-1 knockout mice are more sensitive to hyperpolarization triggered by deprivation. On the contrary, these neurons are more sensitive to hyperpolarization induced by rotenone treatment, but following hyperpolarization knockout neurons are irreversibly depolarized. This led the authors to hypothesize a metabolic roll for DJ-1, possibly with respect to ion gradient maintenance. The membrane potential of neurons is maintained in an ATP dependent manner by the sodium-potassium pump (Na+/K+ pump). When they treated with Oubain, an inihibitor of the Na+/K+ pump, neurons from the knockout mice were hypersensitive to the loss of membrane potential. While they authors propose a metabolic role for DJ-1, they fail to show a mechanism for that role, or physiological effects of DJ-1 loss on any metabolic pathways. It is important to note here that rotenone in addition to causing mitochondrial dysfunction, subsequently induces oxidative stress. The role of oxidative stress in these processes remains unclear.

A wholly separate mouse model was derived by Kim et al. (Kim et al. 2005). Similar to the previous model, these mice were generated using homologous recombination directed to the DJ-1 gene; however this model did not target the second exon of DJ-1, but instead removed exons 3 through 5 of the DJ-1 gene. Furthermore, the researchers mutated residues in exon 2, to establish a premature stop codon in the DJ-1 open reading frame. The result is a DJ-1 mRNA transcript that if translated will only encode the first 8 amino acids of the DJ-1 protein. This small peptide is unstable, and no DJ-1 protein product is present in the resulting mice. Similar to the other DJ-1 knockout mice, these mice do not display a frank Parkinson's disease like phenotype. Also, in agreement with the other papers published using DJ-1 knockout mice, these mice contain

normal numbers of dopaminergic neurons under basal conditions. Kim et al. however went on to examine the effects of oxidative toxin exposures in these mice. Treatment of primary cortical neurons from these mice with hydrogen peroxide showed a 20% increased cell death in the knockout mice compared to their wild type counterparts. Mice heterozygous for the mutant DJ-1 allele showed an intermediate sensitivity to hydrogen peroxide induced cell death, indicating a gene dosage effect. Indeed, this sensitivity is specific to DJ-1 expression and not a strain difference or off target effect, since restoration of DJ-1 expression via infection with a DJ-1 encoding adenovirus protected the neurons from hydrogen peroxide. Furthermore, the authors show that infection with the L166P mutant DJ-1 actually slightly increased neuronal cell death. They suggest that this mutant may have a dominant negative effect on DJ-1. In contrast to the previous knockout mouse study, rotenone treatment in this model did reveal increased cell death of mesencephalic dopaminergic neurons in the absence of DJ-1 expression. DJ-1 knockout neurons were not sensitive to cell killing by non-oxidative treatments including the Topoisomerase I inhibitor camptothecin, or the global kinase inhibitor, staurosporine.

Measuring the movement of wild type and knockout mice in a home cage environment over the course of 24 hours, did not show statistically significant differences in the mice's movement. There was a slight trend toward less movement with respect to DJ-1 genotype, where wild type mice moved slightly more than heterozygous mice, who moved slightly more than knockout mice. Treatment of the mice with the dopaminergic neuronal toxin, MPTP, caused a statistically significant decrease in the movement of knockout mice compared to wild type mice, with heterozygous mice displaying an intermediate phenotype, showing a differential sensitivity to the oxidative toxin with

respect to DJ-1 expression. Conversely, treatment with amphetamine, which, among other things, reverses the dopamine transporter leading to dopamine overflow, and increased movement in mice, induced a smaller increase in the movement of knockout mice than in wild type. This would suggest that, like the previous knockout mouse studies, DJ-1 indeed might have dopaminergic effects beyond cytoprotection. Injection of MPTP into knockout mice caused a greater loss of TH+ dopaminergic neurons than in wild type, and adenoviral add back of DJ-1 reversed this sensitivity compared with contralateral injection of a LacZ encoding adenovirus. These findings suggest, that loss of DJ-1 protein may not have a striking effect on cell survival under normal, controlled, laboratory conditions, but may be essential for cellular protection following exposure to oxidative environmental toxins.

1.9 Detoxification reactions

When challenged with toxic insults, cells respond by activating pathways that lead to protection of the organism. This includes inducing the activity and expression of a gamut of detoxification enzymes (Reviewed in (Wilkinson et al. 1997; Owuor et al. 2002; Hayes et al. 2005)). Systemic detoxification, in general, follows a two step series of detoxification reactions. Phase I detoxification functions to directly neutralize chemical insults to a non-toxic state, or to form activated intermediates that can be further detoxified and or removed from the body by phase II enzymes. It is noteworthy that in many instances these activated intermediates are actually more toxic than their precursors but are a necessary step in removal from the body. If these reactive intermediates are not quickly and adequately processed by phase II detoxification, they can accumulate causing

systemic damage to tissues most notably: mutagenesis, transformation, and cancer (Rooney et al. 2004). The most common and well studied phase I enzymes are the cytochrome P450 family. This large enzyme family has the unique ability to catalyze the oxidation of carbon in un-activated carbon-hydrogen bonds. Such oxidation alters the biological activity of most bioactive organic small molecules including pharmaceuticals and toxins alike. The enzymatic activity of cytochrome P450 enzymes produces oxygen containing free radicals as a byproduct (Yardley-Jones et al. 1991; Rashba-Step et al. 1994). These free radicals are highly toxic to cells, are an important cause of oxidative stress, and can damage DNA leading to cancer (Reviewed in (Karihtala et al. 2007)). These free radicals must therefore be removed as a component of detoxification in addition to the initial toxic compounds. Phase II detoxification is traditionally thought of as a series of conjugation reactions leading to compound excretion. Two examples include glutathione conjugation, which produces water soluble compounds that are excreted by the kidneys into the urine; and glucuronidation, in which glucuronic acid is conjugated with compounds signaling for their excretion into bile and therefore into feces. Another important component of phase II detoxification are the induction of antioxidant responses which remove oxidative species generated by phase I detoxification, and regenerate antioxidant compounds that are depleted during detoxification such as glutathione. The master regulator of these antioxidant responses is the transcription factor Nrf2.

1.10 Nrf2: a master regulator of antioxidant transcription

As a component of phase II detoxification responses, Nuclear factor erythroid 2 – related factor 2 (Nrf2) is activated following oxidative or xenobiotic stresses, as well as by several non-toxic natural mimetics (Reviewed in (Kang et al. 2005)). Nrf2 activates the expression of genes containing an antioxidant response cis-element (ARE) in their promoter (Moi et al. 1994). These genes encode a wide variety of detoxification enzymes such as NAD(P)H quinone oxidoreductase I (NQO1), Heme oxygenase-1 (Hmox1), and Epoxide hydrolase (Ephx). These enzymes function to detoxify oxidative compounds, remove reactive oxygen, and to regenerate antioxidant compounds present in cells such as tocopherols like vitamin E (Nioi et al. 2004). Enzymes governing the generation and recycling of glutathione are also prototypic gene targets of Nrf2 regulated transcription. These include glutathione cysteine ligases, glutathione-S-reductases and glutathione-S-transferases, which are key pathway components responsible for maintaining adequate levels of reduced glutathione in cells thereby maintaining normal oxidative conditions. For a list of Nrf2 regulated genes see Table 1.2.

Dysregulation of Nrf2 and dysfunction of Nrf2 regulated genes have been implicated in the risk for cancer and neurodegenerative diseases (Smith 1999; Harada et al. 2001; Shen et al. 2005; van Muiswinkel et al. 2005; Yu et al. 2005). For example, epoxide hydrolase (Ephx) gene polymorphisms that decrease the enzyme's efficiency are common. Carriers of these polymorphisms who smoke have a 10-fold higher risk of developing lung cancer than cigarette smokers who do not carry the gene polymorphism (Benhamou et al. 1998). This clinical correlate is striking both because lung cancer

caused by cigarette smoking is so prevalent, and because Ephx is only one of several genes controlled by Nrf2 that can act to breakdown cyclic oxidative compounds such as epoxides.

NAD(P)H quinone oxidoreductase 1 (NQO1) is a prototypic Nrf2 regulated gene and provides a good model to consider Nrf2 activity. This is due to the fact that NQO1 is promiscuous in its ability to reduce cyclic substrates (Faig et al. 2000), and because NQO1 activity is largely regulated by its expression, which is controlled by Nrf2 (Jaiswal 2000).

NQO1 catalyzes an obligate two-electron reduction of cyclic compounds. Similar to oxidation by cytochrome P450 enzymes, this obligate reduction can detoxify cyclic compounds or activate them increasing reactivity and/or toxicity (Winski et al. 2001). The clearest example of the importance of NQO1 activity is cancer prevention and its central role in benzene detoxification. Polymorphisms in the *nqo1* gene render individuals' bone marrow susceptible to benzene toxicity (Moran et al. 1999). Likewise, decreased NQO1 activity and/or expression are associated with myelogenous hyperplasia and leukemias (Long et al. 2002; Iskander et al. 2005; Das et al. 2006). The role of NQO1 in neurodegenerative diseases has been a major focus of recent research, and NQO1 associations with Parkinson's disease have been proposed (Harada et al. 2001; van Muiswinkel et al. 2004).

1.11 Regulation of Nrf2 activity

Nrf2 is a Cap 'n' collar transcription factor that regulates the expression of a large gamut of detoxification enzymes in response to oxidative stress and xenobiotic

compounds (Kang et al. 2005). Nrf2 function is tightly regulated so as to allow for fast and robust gene induction in response to toxic stresses. Prior to toxic insult, Nrf2 is maintained in a basal state bound to its cytosolic inhibitor protein, Kelch-like ECH-associated protein 1 (Keap1) (Itoh et al. 1999). Keap1 protein binds Nrf2 via its Kelch domain, and at the opposite end of the protein, Keap1 binds Cullin-3 (Cul3) via a BTB/POZ domain. The Keap1(BTB)-Cul3-Roc1 protein complex promotes ubiquitination of Nrf2, which is then degraded by the 26S proteosome (Kobayashi et al. 2004; Zhang et al. 2004; Furukawa et al. 2005). This constitutive degradation of Nrf2 in the absence of toxic stress, represses Nrf2 driven gene transcription while maintaining an active-ready pool of Nrf2 protein, that is constantly being translated, prepared for activation in the event of toxic or oxidative stresses.

When cells are exposed to oxidative stress, xenobiotics, or oxidant mimetics, cellular sensors respond by causing Nrf2 to dissociate from Keap1, and Nrf2's nuclear localization signal (NLS) is unmasked. Nrf2, now free from ubiquitination and degradation, is stabilized and can translocate to the nucleus strongly inducing the expression of genes containing antioxidant response elements with their promoters. This mechanism of activation is parallel to, and can be compared with, the activation of the hypoxia inducible factor 1 alpha and 2 alpha (HIF1α /HIF2α). These factors are maintained under normoxic conditions bound in the cytosol to their inhibitor protein, the Von Hippel-Lindau tumor suppressor (VHL). VHL, like Keap1, is part of a ubiquitin ligase complex composed Cullin-2 and Ring-box 1 (Rbx1) instead of Cul3-Roc1 (Maxwell et al. 1999; Cockman et al. 2000). And like Keap1, VHL constitutively targets HIF1α /HIF2α for ubiquitination and subsequent degradation under basal conditions,

maintaining an active-ready pool of HIF protein in the event of hypoxia. (See Figure 1.3 for an overview) It is perhaps noteworthy that the activation signals of both of these two important yet distinct systems rely on oxygenation for activation. In the case of Keap1-Nrf2, it is the excess of oxygen – in the form of free radicals, or hyperoxia, that activates the stabilization and induction of Nrf2; while conversely it is the absence of oxygen – or hypoxia, which activates the stabilization and induction of HIF regulated transcription. This is an example of convergent evolution, in which similar molecular mechanisms govern the function of these two distinct, and unrelated transcription factors.

The cellular sensors that recognize oxidative stress and induce Nrf2 stabilization remain unclear and controversial. It has been proposed that Keap1 itself is a cellular sensor of oxidative stress (Dinkova-Kostova et al. 2002). Indeed, there are thiol rich regions of the linker domain joining the BTB and Kelch domains of Keap1, that can be oxidized and physiologically attainable redox potentials (Dinkova-Kostova et al. 2002). However, to date, Keap1 has not been shown to be oxidized *in vivo*. And, while mutation of cysteines at positions 273 and 288 within Keap1 disrupt the ubiquitination of Nrf2, they do not affect NLS masking of Nrf2 by Keap1, since the Keap1-Nrf2 complex is not disrupted in these mutations (Kobayashi et al. 2006). Furthermore, multiple signaling cascades including: PI3K/Akt, SAPK/p38, MAPK/Erk, and JNK have all been shown to be required for Nrf2 activation following various oxidative/mimetic signals (Alam et al. 2000; Zipper et al. 2000; Nakaso et al. 2003; Zipper et al. 2003; Martin et al. 2004; Wu et al. 2006; Yuan et al. 2006).

1.12 Conclusions

Functional studies of DJ-1 in cell culture, drosophila, mouse, and human models have shown that DJ-1 is a pro-survival protein, that functions to protect cells from cytotoxic insults. However, the mechanism by which DJ-1 performs this function in cells has remained elusive. DJ-1 is strongly linked to cancer, and the loss of DJ-1 causes Parkinson's disease. Restoring DJ-1 expression to these Parkinson's disease patients lacking DJ-1 would likely prevent their disease. Unfortunately, gene therapy is problematic, especially gene transfer to neurons in the brain. Likewise, the reduction of DJ-1 expression in cancer cells renders them susceptible to cell killing, and DJ-1 expression correlates inversely with patient prognosis. Therefore, understanding the mechanism of DJ-1 cytoprotection is essential: both to understand the biological underpinnings of cancer and Parkinson's disease, and to identify targets of therapy to mediate DJ-1 function.

Figure 1.1: Schematic of the neuronal connections in the brain affected during Parkinson's disease. This schematic represents the motor control pathways disrupted dueing Parkinson's disease. The primary deficit in Parkinson's disease is the loss of dopaminergic neurons within the Substantia Nigra. These neurons extend their axons to the Caudate and Putamen. The net effect of the dopaminergic neurons in the Substantia Nigra is to inhibit motor neurons in the cortex. Therefore, when the dopaminergic neurons are lost, as in Parkinson's disease, the motor cortex is over-active, leading to rigidity and loss of motor coordination.

Figure 1.1

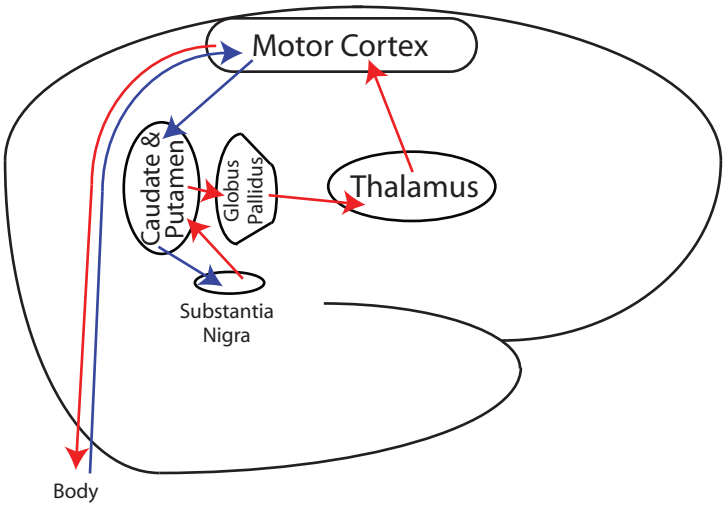

Motor Cortex
Caudate & Putamen
Globus Pallidus
Thalamus
Substantia Nigra
Body

Table 1.2: Nrf2 regulated genes. This table summarizes the genes regulated by the antioxidant transcription factor Nrf2. Gene symbol, common name, and function are all indicated. Nrf2 induces genes that either directly detoxify oxidative species or maintain antioxidant compounds in the cell such as glutathione.

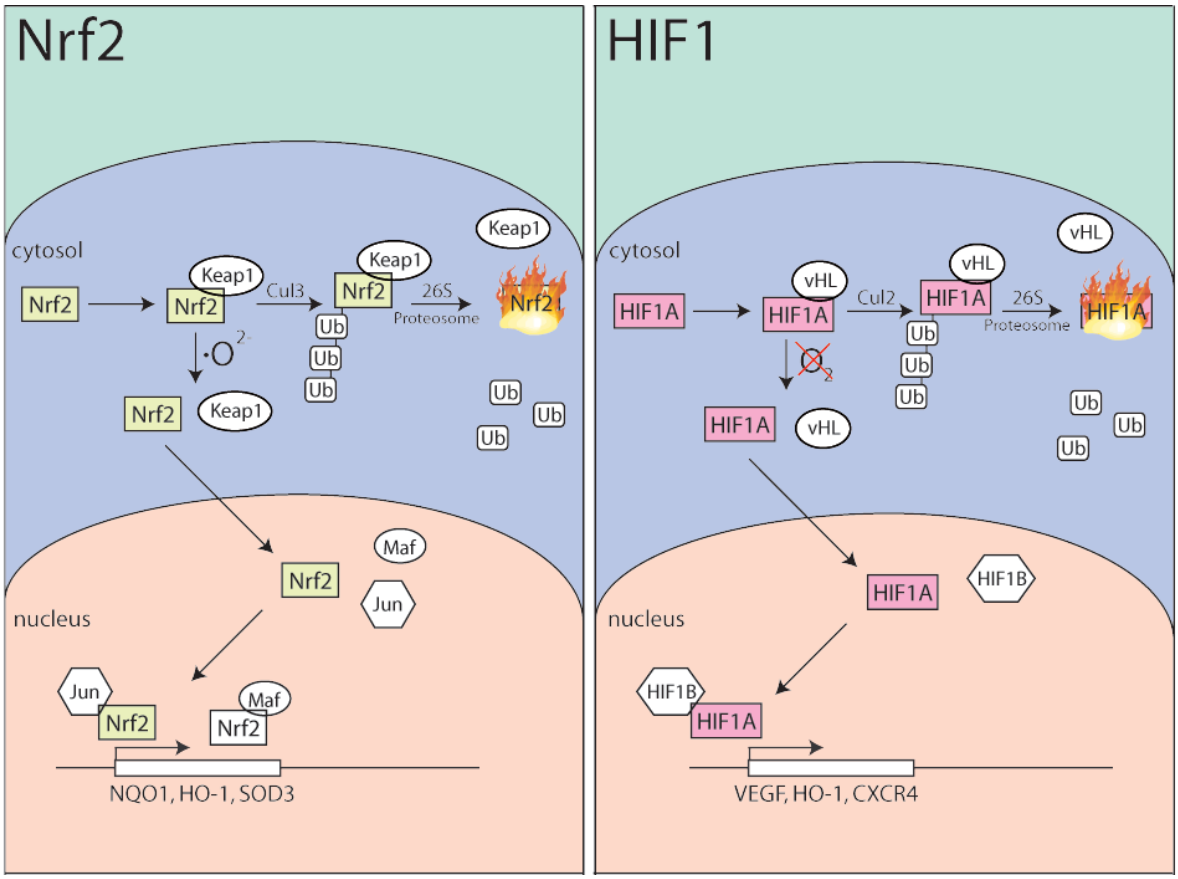
Table 1.2

Nrf2 regulated genes:

Gene:	**Common name:**	**Function:**
Nqo1	NAD(P)H Quinone Oxidoreductase-1	2 election reduction of cyclic molecules
Hmox-1	Heme Oxygenase-1	detoxification of oxidative species
Gcl (m&c)	Glutathione cysteine ligase (both subunits)	Glutathione metabolism
GST (several)	Glutathione-S-transferase	Glutathione metabolism
Gpx (several)	Glutatione peroxidase	Glutathione metabolism
Sod3	Superoxide dismutase-3	Detoxification of superoxide
Txn	Thioredoxin	disulfide reductase

Figure 1.3: Comparison of Nrf2 regulation with HIF1α . Both Nrf2 and HIF1α are maintained in a basal state in the cytosol bound to an inhibitor protein that targets the transcription factor for constitutive ubiquitination and subsequent degradation. In the case of Nrf2, Keap1 targets Nrf2 for ubiquitination in a Cullin-3 dependent manner, while with HIF1α is bound to the von Hippel Lindau tumor suppressor that targets HIF1α for ubiquitination by Cullin-2. Nrf2 is activated by oxidative stress, while HIF1α is activated by hypoxia, the absence of oxygen. Following activation, the transcription factors both translocate to the nucleus where they form heterodimers with other factors already present, and activate the transcription of a genes that protect cells from stress. It is noteworthy that these two transcription factors induce some common genes, including Hmox-1, that function to maintain oxygen homeostasis in the cell.

Figure 1.3


Nrf2
cytosol
Nrf2
Keap1
Nrf2
Cul3
Keap1
Nrf2
26S
Proteosome
Keap1
Nrf2
Ub
Ub
Ub
Ub
Ub
Ub
Nrf2
Keap1
Maf
Nrf2
Jun
nucleus
Jun
Nrf2
Maf
Nrf2
NQO1, HO-1, SOD3
HIF1
cytosol
HIF1A
vHL
HIF1A
Cul2
vHL
HIF1A
26S
Proteosome
vHL
HIF1A
Ub
Ub
Ub
Ub
Ub
Ub
HIF1A
vHL
HIF1A
HIF1B
nucleus
HIF1B
HIF1A
VEGF, HO-1, CXCR4

CHAPTER II:

DJ-1/PARK 7 stabilizes the anti-oxidant transcriptional master regulator, Nrf2: Implications in cancer and Parkinson’s disease.

2.1 ABSTRACT:

DJ-1/PARK7, a cancer and Parkinson's disease associated protein, protects cells from toxic stresses. However, the functional basis of this protection has remained elusive. We found that loss of DJ-1 leads to deficits in NQO1, a detoxification enzyme. This deficit is attributed to a loss of Nrf2, a master-regulator of anti-oxidant transcriptional responses. DJ-1 stabilizes Nrf2 by preventing association with its inhibitor protein, Keap1, and Nrf2's subsequent ubiquitination. Without intact DJ-1, Nrf2 protein is unstable and transcriptional responses are thereby decreased both basally and following induction. This effect of DJ-1 on Nrf2 is present in both transformed lines and primary cells, and across human and mouse species. DJ-1's function on Nrf2 and subsequent effects on antioxidant responses may explain how DJ-1 affects the etiology of both cancer and Parkinson's disease, seemingly disparate disorders. Furthermore, this DJ-1/Nrf2 functional axis presents a therapeutic target in cancer, and justifies DJ-1 as a tumor biomarker.

2.2 INTRODUCTION:

Oxidative stress has been implicated as a major contributing factor to a wide variety of ailments. Cancer, cardiovascular disease, neurodegenerative disorders, and aging are all associated with increased oxidative stress in tissues. Such stress results from the accumulation of oxidative species due to their metabolic generation and environmental exposures. These oxidative species are detoxified by a gamut of antioxidant enzymes and molecules. The balance between oxidative species generation and removal determines the oxidative stress on a given tissue. Not surprisingly therefore, cellular responses to oxidative stress are major determinants of disease susceptibility. This is particularly true in tissues that are sensitive to oxidative stress such as the central nervous system. Genetic defects in oxidative responses lead to neurodegenerative diseases. Examples include mutations in SOD that lead to amyotrophic lateral sclerosis (ALS) (Beckman et al. 1993), and more recently, loss of DJ-1, which leads to early onset Parkinson ’s disease with high penetrance (Bonifati et al. 2003).

DJ-1 was initially described as a putative oncogene, able to transform cells weakly on its own and more strongly in combination with Ras (Nagakubo et al. 1997). DJ-1 is expressed at high levels in primary lung and prostate cancer biopsies (MacKeigan et al. 2003; Hod 2004) and its expression correlates negatively with clinical outcomes in non-small cell lung carcinoma patients (Kim et al. 2005). The DJ-1 protein affects cell survival, in part, by modulating cellular signaling cascades such as PTEN/PI3K/Akt (Kim et al. 2005) and altering p53 activity (Shinbo et al. 2005). Additionally, we and others have previously shown that DJ-1 expression in cancer cell lines conveys protection against stresses including: chemotherapy, oxidative stress, ER stress, and proteosome inhibition (MacKeigan et al. 2003; Yokota et al. 2003; Taira et al. 2004). The

mechanism by which DJ-1 imparts this protection remains unknown. We report here that DJ-1 is required for the activity of Nrf2, a master regulator of response to oxidative stress.

Nrf2 is a member of the Cap ‘n’ Collar (CNC) family of b-Zip transcription factors that regulate the expression of many antioxidant pathway genes (reviewed in (Cho et al. 2006)). Nrf2 is maintained at basal levels in cells by binding to its inhibitor protein, Keap1 (Itoh et al. 1999; Dhakshinamoorthy et al. 2001). Keap1 is a BTB domain containing protein that targets Nrf2 for ubiquitination by Cul3/Roc-1 leading to its constitutive degradation (Cullinan et al. 2004; Kobayashi et al. 2004; Zhang et al. 2004; Furukawa et al. 2005). Upon exposure to oxidative stress, xenobiotics, or electrophilic compounds, Nrf2 protein is stabilized and translocates to the nucleus (Chen et al. 2004). There it forms heterodimers with other transcription regulators, such as small Maf proteins, and induces the expression of antioxidant genes (Itoh et al. 1997; Wild et al. 1999). Nrf2 drives the expression of detoxification enzymes such as NQO1, Hmox-1, and enzymes that generate antioxidant molecules such as glutathione (Venugopal et al. 1996; Alam et al. 1999). Nrf2 function and the expression of its regulated genes, including NQO1, have been implicated in the risk and/or prevention of both cancer and Parkinson’s disease (Ramos-Gomez et al. 2001; Fahey et al. 2002; Kwak et al. 2003; Cao et al. 2005; Nakaso et al. 2006; Satoh et al. 2006).

This study finds that DJ-1 is required for the expression of several genes including the prototypic Nrf2 regulated antioxidant enzyme: NQO1. We report here that DJ-1 is indispensable for Nrf2 stabilization by affecting Nrf2 association with Keap1, an inhibitor protein that promotes the ubiquitination and degradation of Nrf2. This implicates DJ-1’s effects on Nrf2 in the development of Parkinson’s disease and cancer, and represents potential novel therapeutic targets.

2.3 RESULTS:

siRNA mediated knockdown of DJ-1 and Affymetrix GeneChip® analysis

To explore DJ-1's function, we examined its effect on global gene expression. DJ-1 expression was reduced by small interfering RNA (siRNA) in H157 non-small cell lung carcinoma cells (Figure 2.1 A, B and C). The characterization of the antibody used to verify DJ-1 expression is shown in Figure 2.2. The first DJ-1 siRNA (referred to as siDJ-1#1) caused modest decrease of DJ-1 while siDJ-1#2 caused profound decrease. RNA samples from cells with siDJ-1#1, siDJ-1#2, two control scrambled oligomers (siCTL) and one mock transfected sample were subjected to Affymetrix GeneChip profiling. To ensure that changes warranted further study, we stringently filtered expression to exclude differences less than three fold, and any genes having spots with a raw signal intensity of less than 500 units in the samples where a gene was determined to be present. This stringent filtering produced a list of three genes that were increased and fourteen genes that were decreased in cells with siDJ-1 (Figure 2.3). As expected, siDJ-1 reduced DJ-1 expression.

Among the genes whose expression decreased in the absence of DJ-1 one of particular interest was NAD(P)H Quinone Oxidoreductase 1 (NQO1). NQO1 is a well described detoxification enzyme (Iyanagi et al. 1970) that has been implicated in the risk and prevention of cancer and neurodegenerative diseases (Cresteil et al. 1991; Kolesar et al. 1995; Hara et al. 2003; van Muiswinkel et al. 2004). NQO1 is regulated to a large degree by gene transcription via an anti-oxidant response element (ARE) in its promoter (Jaiswal 2000), which is a prototypic target of the antioxidant transcription factor, Nrf2 (Venugopal et al. 1996). With this in mind, we used the tfsearch algorithm (as in (Kast et al. 2003)) to search for putative ARE within 1,000 base pairs

upstream of the transcriptional start site of the genes identified in Figure 2.3. Seven out of seventeen genes that were changed by more than 3 fold by siDJ-1 contained an ARE-like sequence (TMAnnRTGAYnnnGCRwwww) in their promoters (Figure 2.3, last column). We then re-analyzed our microarray data with respect to Nrf2 and found that several Nrf2 regulated genes were altered in the absence of DJ-1 (Figure 2.4). All array data is deposited in the GEO online repository, series number GSE5519.

DJ-1 is required for Nrf2 mediated transcription

To verify the microarray data, we used NQO1 as a prototypic target gene of DJ-1. Real-time PCR analysis shows that siDJ-1#2 reduced DJ-1 and NQO1 by over 80%. However Nrf2 mRNA expression was not changed (Figure 2.5A). This indicates that NQO1 expression differences are not due to a reduction of Nrf2 mRNA. To determine if DJ-1 affects NQO1 gene transcription via Nrf2 function, we utilized a reporter construct, pGL2-ARE which contains the firefly luciferase gene under the control of ARE from the human NQO1 promoter (Figure 2.5B). This construct was tested in the absence or presence of DJ-1. The liver cell line Huh7 was used since Nrf2 activity can be induced in these cells by the non-toxic food preservative, tert-butylhydroquinone (tBHQ) (Huang et al. 2000). Cells were treated with either 50 μM tBHQ or DMSO vehicle control and luciferase activity measured (Figure 2.5B). Flag-Nrf2 was transfected into cells as a positive control. Over-expressed Nrf2 robustly activated ARE-regulated luciferase (lanes 1 vs. 2). Cells with siCTL produced a basal level of luciferase, while tBHQ induced luciferase expression as expected (Dhakshinamoorthy et al. 2002) (lanes 3 and 4). In the presence of siDJ-1#1 or siDJ-1#2, luciferase activity was reduced (lanes 5 and 7), and it was no longer stimulated by tBHQ treatment (lanes 6

and 8). This effect is specific for the ARE element, as siDJ-1 did not affect other promoter elements (Figure 2.5C-2.5D).

DJ-1 is required for Nrf2 protein stability

Given that DJ-1 was required for both basal and induced ARE-driven transcription, we explored some possible mechanisms. DJ-1 was not associated with the NQO1 promoter as assessed by chromatin immunoprecipitation assay, suggesting that DJ-1 is not likely tethered on the NQO1 promoter with Nrf2 (Figure 2.6). Furthermore, RNA expression of Nrf2 (see Figure 2.5A) or its inhibitor, Keap1 were not changed by siDJ-1 (Figure 2.7). However, western blot analysis revealed that Nrf2 protein expression was drastically reduced in the absence of DJ-1, with siDJ-1#1 causing a more modest decrease, and siDJ-1#2 causing a dramatic decrease (Figure 2.8A), consistent with the level of DJ-1 reduction achieved with these two siRNA (see Figure 2.1A).

To determine if DJ-1 reduced Nrf2 stability, DJ-1 was decreased by siDJ-1 in Huh7 cells, and the cells were treated with the translation inhibitor, cyclohexamide (CHX) to prevent new protein synthesis. Cells were lysed at various timepoints and the degradation kinetics of Nrf2 and, as a control, actin was analyzed by western blot. Nrf2 protein was decreased by siDJ-1 compared to siCTL or transfection reagents alone, and by 90 minutes, Nrf2 disappeared in cells with siDJ-1 (Figure 2.8B). This indicates that DJ-1 stabilizes Nrf-2 protein.

Nrf2 protein stability is an important regulatory event, which is tightly controlled by its association with a cytosolic inhibitor protein, Keap1 (Itoh et al. 1999; Dhakshinamoorthy et al. 2001). Under unstimulated conditions, Nrf2 associates with Keap1 which targets Nrf2 for ubiquitination by a Cullin-3 dependent mechanism (Cullinan et al. 2004; Kobayashi et al. 2004; Zhang et al. 2004; Furukawa et al. 2005), leading to proteosome-dependent degradation (Stewart et

al. 2003). Given our data implicating DJ-1 in Nrf2 stability, we tested DJ-1's effect on Nrf2 ubiquitination (Figure 2.8C). Huh7 cells expressing HA-tagged ubiquitin and Nrf2 were transfected with DJ-1 or pcDNA. Nrf2 was immuno-precipitated from denatured lysates, isolating only molecules covalently linked to Nrf2. Ubiquitin-Nrf2 conjugates were visualized by immunoblotting for the ubiquitin epitope, HA. Nrf2 was ubiquitinated to a much lesser degree when DJ-1 was over-expressed (Figure 2.8C, top panel, lanes 1, 2). This is correlated with an increase of Nrf-2 protein in the presence of DJ-1 (lower panel). The addition of the proteosome inhibitor MG132 prevented degradation of ubiquitinated Nrf2 (lanes 5 and 6).

Given that the association of Keap1 with Nrf2 is known to trigger Nrf2 ubiquitination/degradation (McMahon et al. 2003) while DJ-1 reduces Nrf2 ubiquitination, we determined if DJ-1 affects the association of Nrf2 and Keap1. Huh7 cells were transfected with V5-tagged Keap1; the tagged-epitope is required due to the lack of a sufficient and commercially available Keap1 antibody. The anti-V5 antibody recognized V5-Keap1 and co-immunoprecipated Nrf2 (Figure 2.8D, lane 1); the inclusion of Flag-DJ-1 eliminated this co-immunoprecipitation (Figure 2.8D, lane 2). Reverse immuno-precipitation shows that antibody to endogenous Nrf2 co-precipitated V5-Keap1 (Figure 2.8D, lane 4), which was also decreased by Flag-DJ-1 (lane 5). These data suggest that DJ-1 stabilizes Nrf2 by preventing its association with Keap1.

While the above experiments demonstrate a strong functional link between DJ-1 and Nrf2, we have so far been unable to determine where DJ-1 physically exerts this effect. Co-immunoprecipitation experiments have failed to find DJ-1 in physical association with Nrf2, Keap1, or Cullin-3 (Figure 2.9). Therefore, it remains to be determined if DJ-1's profound effect on Nrf2 is the result of direct or indirect molecular mechanisms.

DJ-1 is required for Nrf2 function in primary mouse embryonic fibroblasts (MEFs)

To determine if DJ-1 is required for Nrf2 expression in primary untransformed cells, we isolated day 13.5 mouse embryonic fibroblasts (MEFs) from DJ-$1^{-/-}$ mice (Kim et al. 2005), and induced Nrf2 protein expression using tBHQ treatment. tBHQ induced mNrf2 protein expression in wildtype littermates (n=4, two shown here), while DJ-$1^{-/-}$ mice failed to show induced mNrf2 expression (n=4, two shown here) (Figure 2.10A). Restoration of DJ-1 with a flag-DJ-1 expression plasmid also restored Nrf2 protein expression with tBHQ treatment (Figure 2.10B). This indicates that the loss of Nrf2 protein in DJ-$1^{-/-}$ fibroblasts is a specific consequence of the loss of DJ-1.

To examine the necessity of DJ-1 for Nrf2 function, we resorted to the Nrf2-activated reporter plasmid, pGL2-ARE. DJ-$1^{+/+}$ and DJ-$1^{-/-}$ MEFs from four mice (two representatives are shown in Figure 2.10C) were separately transfected with pGL2-ARE and then induced with 50 μM tBHQ. Wild-type DJ-$1^{+/+}$ cells showed increased luciferase expression upon tBHQ treatment, while DJ-$1^{-/-}$ cells did not (Figure 2.10C, top panel). SV40 promoter activity was independent of DJ-1 (Figure 2.10C, bottom).

To use a more physiologic measurement, the effect of DJ-1 on the expression of Nrf2-regulated detoxification enzymes: NQO1 and Glutathione Cysteine Ligase, modifier subunit (GCLM) was tested (Figure 2.10D). Based on the microarray analysis (Figure 2.4), siDJ-1 reduced GCLM expression by 1.478 fold, hence we selected it in addition to NQO1 for further analysis. Induction of MEF cultures with 25 μM tBHQ led to a substantial increase of mNQO1 in DJ-$1^{+/+}$ MEFs but this increase was drastically reduced in DJ-$1^{-/-}$ cells. This pattern is also found for mGCLM. However, at higher (100 μM) dosage, even though differences in mNrf2 protein expression persisted (Figure 2.10A), induction of detoxification enzymes is only slightly reduced in

DJ-$1^{-/-}$ compared to DJ-$1^{+/+}$ MEFs (Figure 2.11) indicating that high concentration of tBHQ can activate a DJ-1 independent pathway to cause NQO1 and GCLM expression.

2.4 DISCUSSION:

In summary, this work describes functional effects of the DJ-1 protein via Nrf2, a master regulator of anti-oxidant gene responses. Cancer and PD lie at opposite ends of a spectrum defined by dysfunctions in cell death. Our finding may explain how DJ-1 plays an important role in both diseases. One of the hallmarks of PD is the loss of *substantia nigra* dopaminergic neurons, leading to motor deficits (Kish et al. 1988). DJ-$1^{-/-}$ mice did not exhibit widespread neuronal loss in a PD disease model (Goldberg et al. 2005; Kim et al. 2005), but these neurons were more susceptible to death following toxic insults (Kim et al. 2005). Likewise, human neuronal cell lines with DJ-1 knockdown are more sensitive to toxic compounds (Yokota et al. 2003; Taira et al. 2004). The loss of anti-oxidant gene transcription could account for these phenotypes that are only evident after environmental harm.

It is noteworthy that we initially identified DJ-1's effect on Nrf2 in lung tumor cells. Studies of Nrf2 knockout mice show that it plays a significant role in lung biology (reviewed in (Cho et al. 2006)). In our studies we found that the H157 lung tumor cells did not consistently induce Nrf2 activity following tBHQ treatment, but instead had a very high basal level of activity that was not inducible by treatment (data not shown). High basal NQO1 expression allowed us to confidently quantify changes in NQO1 expression and implicated the broader effect of DJ-1 on Nrf2. In order to study gene induction, we then used liver cell line models, which are highly inducible. These models allowed us to first identify the effects of DJ-1 on Nrf2 that has heretofore remained unrecognized.

Enhanced expression of DJ-1 in cancer cells leading to increased detoxification enzymes is likely to provide a survival advantage. However, these enzymes may be exploited as novel

treatment targets in tumors. For example, NQO1, an obligate two-electron reductase, can reduce anti-tumor quinones leading to their bioactivation. Mitomycin C (MMC), and a novel anti-tumor compound, 2,5-diaziridinyl-3- (hydroxymethyl)-6-methyl-1,4-benzoquinone, are activated by NQO1 activity and NQO1 is shown to increase the efficacy of MMC *in vivo* (Begleiter et al. 2004). It is possible that tumors with high DJ-1 might be more susceptible to therapies that rely on enzymes such as NQO1. This underscores the potential of DJ-1 as a biomarker to define specific anti-tumor therapies.

2.5 MATERIALS AND METHODS:

Cell culture, treatments, and plasmid constructs - Huh7 cells were grown in DMEM (Sigma) with 7% FCS. H157 cells were grown in RPMI-1640 (Gibco) +10% FCS. All mammalian cell culture was grown in the presence of penicillin and streptomycin to minimize contamination effects.

Tert-Butylhydroquinone (Fluka) was dissolved in DMSO (final concentration on cells of 0.0001%) and cells were treated for 18-24 hours. Dexamethasone and forskolin (MP Biochemicals) were dissolved in DMSO and ethanol respectively. Dexamethasone was used at a final concentration of 100 μM, and forskolin at 10 μM. In experiments determining Nrf2 protein stability, cells were treated with cyclohexamide (Sigma) in DMSO at a concentration of 75 μg/mL for up to two (2) hours. The peptide proteosome inhibitor MG132 (Calbiochem), was used at 25 μM for 4-6 hours for ubiquitination studies.

Other investigators generously provided flag-DJ-1 (Hod 2004), flag-Nrf2 (Furukawa et al. 2005), and hNQO1-ARE-pGL2 (Dhakshinamoorthy et al. 2000) plasmids. SV40-Luciferase [pGL3-control], GRE-Luciferase [pGRE-Luc] (Clontech), and CRE-Luciferase [pCRE-Luc] (Clontech) were all purchased from commercial sources. We directionally cloned human Keap1 into the V5/His containing pcDNA3.1D-Topo plasmid (Invitrogen) by amplifying the Keap1 ORF with the primers 5' CACCATGCAGCCAGATCCCAGGCCTAGC 3' and 5' ACAGGTACAGTTCTGCTGGTCAATCT 3' using platinum-*pfx* polymerase (Invitrogen). Clone directionality and expression was verified by sequencing and western blot analysis. Human cell lines were transfected with DNA using Fugene6 (Roche) and MEF cultures were transfected with Lipofectamine 2000 (Invitrogen) per manufacturer instructions.

siRNA knockdown of DJ-1 – Cell lines were transfected with siDJ-1#1 5' NNGACCCAGUACAGUGUAGCC 3', siDJ-1#2 5' NNUGGAGACGGUCAUCCCUGU 3', scrambled control oligomer (Xeragon), siCONTROL (siCTL) non-targetting siRNA #1 (Dharmacon), or transfection reagent alone (siMock) using Oligofectamine (Invitrogen) for H157 cells, or Lipofectamine 2000 (Invitrogen) for Huh7 cells as per manufacturers protocols. Cells were transfected on consecutive days for 2-3 days in a row, and lysates were taken for RNA and protein analysis ninety-six (96) hours after the first transfection.

Generation of anti-DJ-1 antibody – DJ-1 was cloned into 6x Histidine-tagged *E. coli* over-expression vector, QE82L (Qiagen), by standard methodology. Expression of DJ-1 was induced with 1 mM IPTG (Isopropyl-β-D-Thiogalactopyranoside) in the *E. coli* strain BL21 (DE3). Cells were lysed in PBS plus EDTA-free protease inhibitor cocktail (Roche) and DJ-1 was purified to greater than 95% homogeneity with Ni-NTA (Qiagen) according to the manufacturer's instructions. Recombinant DJ-1 was sent to Proteintech Group Inc. for the production of the anti-DJ-1 rabbit polyclonal serum.

Affymetrix GeneChip® analysis – Total RNA isolated from H157 cells, was DNAse I treated and column purified (Promega). The quality of the RNA was determined by formamide-agarose electrophoresis, and comparison of expression of housekeeping genes. Seven μg of total RNA was used to synthesize cDNA. A custom cDNA kit from Life Technologies was used with a T7- $(dT)_{24}$ primer for this reaction. Biotinylated cRNA was then generated from the cDNA reaction using the BioArray High Yield RNA Transcript Kit. The cRNA was then fragmented in fragmentation buffer

(5X fragmentation buffer: 200mM Tris-acetate, pH8.1, 500mM KOAc, 150mM MgOAc) at 94^{o}C for 35 minutes before the chip hybridization. 15 μg of fragmented cRNA was then added to hybridization cocktail (0.05 μg/μl fragmented cRNA, 50 pM control oligonucleotide B2, *BioB*, *BioC*, *BioD*, and *cre* hybridization controls, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, 100mM MES, 1M [Na^{+}], 20mM EDTA, 0.01% Tween 20). Ten μg of cRNA was used for hybridization. Arrays were hybridized for 16 hours at 45^{o}C in the GeneChip Hybridization Oven 640. The arrays were washed and stained with R-phycoerythrin streptavidin in the GeneChip Fluidics Station 400. After this, the arrays were scanned with the Hewlett Packard GeneArray Scanner. Affymetrix GeneChip Microarray Suite 5.0 software was used for washing, scanning, and basic analysis. Sample quality was assessed by examination of 3' to 5' intensity ratios of certain genes.

These data were then further analyzed, filtered, and compared using GeneSpring software (Silicon Genetics). Gene defined at 'changed' were filtered to include those differing greater than three (3) fold between both siCTL chips and siDJ-1 chips, with a raw fluorescence intensity of at least five hundred (500) in both of the highly-expressed (present) arrays. Both siDJ-1 arrays were transfected with siDJ-1 #1, and then verified by real-time PCR using both siDJ-1 #1 and siDJ-1 #2.

Realtime quantitative PCR – Reactions were carried out in an ABI 7900HT (Applied Biosciences) using 15 μl, 384 well format and master-mixes from ABGene. Taqman PCR primer/probe sets were designed for human DJ-1: primer1 5' CCATATGATGTGGTGGTTCTAC 3', primer2 5' ACTTCCACAACCTATTTCATGAG 3', probe 5' [6-FAM]ACCTGCACAGATGGCGGCTATCA[Tamra-Q] 3' and for human NQO1: primer1 5' CCGTGGATCCCTTGCAGAGA 3', primer2 5' AGGACCCTTCCGGAGTAAGA 3', probe 5' [6-

FAM]ACATGGAGCCACTGCCACCA[Tamra-Q] 3'. SYBR green realtime PCR primers were designed for human Nrf2: primer1 5' AGTGGATCTGCCAACTACTC 3', primer2 5' CATCTACAAACGGGAATGTCTG 3'. We used previously published mouse G3PDH primers designed to be used with SYBR green quantitation (Engelbrecht et al. 2003). Pre-designed Taqman PCR primer and probe sets were purchased from Applied Biosystems for mouse NQO1, and GCLM.

Luciferase reporter gene assays – Cells were grown and transfected, as described above, in 6-well plates (Falcon). Cultures were lysed in reporter lysis buffer (Promega) using a single round of freeze-thaw at -80 C. Luciferase assays were then performed as previously described (Piskurich et al. 1999).

Western blot analysis and immunoprecipitation – For all western blot analyses, cells were lysed in RIPA buffer (10 mM NaPO4 ph 7.4, 300 mM NaCl, 0.1% SDS, 1% NP-40, 1% deoxycholic acid, 2 mM EDTA) with protease inhibitors (Roche), diluted with SDS loading buffer and boiled in the presence of the reducing agent dithiothreitol (DTT). Proteins were then separated by molecular weight using SDS-PAGE through polyacrylimide gels ranging from 6% - 12%. Proteins were electrophoretically transferred to nitrocellulose membranes, and blocked using 5% non-fat dry milk in TBS with 0.1% Tween 20. Antibodies used for blotting were anti-Nrf2 H-300 (Santa Cruz Biotechnology), rabbit polyclonal anti-DJ-1, anti-Actin-HRP (Santa Cruz Biotechnology), anti-G3PDH, anti-Hemagglutanin (HA)-HRP (Roche Diagnostics), and anti-Flag (M2)-HRP (Sigma).

Protein complexes were isolated from cell lysates by immunoprecipitation using antibodies specific for Nrf2 [H-300] (Santa Cruz Biotechnology) and anti-V5 (Invitrogen) followed by

incubation with Protein A/G agarose (Pierce Biotechnologies). Protein A/G-antibody-protein complexes were washed extensively and eluted by boiling in loading buffer with reducing equivalents. Eluates and input lysate controls were then western blotted to assay for protein expression and isolation.

Ubiquitination assays were performed in Huh7 cells transfected with epitope tagged Nrf2 and Ubiquitin grown in 100 mm^2 plates. The cells were lysed in 200 µl SDS lysis buffer (50 mM Tris-HCl pH7.5, 0.5 mM EDTA, 1% SDS, 1 mM DTT) and boiled for 10 minutes. Cellular debris was pelleted and SDS concentrations were diluted by the addition of 1200 µl 0.5% NP-40 lysis buffer with added protease inhibitors. Anti-Flag (M2) agarose was then added and incubated for 14-16 hours. The agarose matrix was washed extensively with 0.5% NP-40 lysis buffer and the proteins were eluted by boiling in 2x loading buffer with DTT. The eluates were then analyzed by western blot analysis for the expression of the epitope tags.

DJ-1 Knockout mice and embryonic fibroblast culture: DJ-1 knockout mice and wild type littermates (Kim et al. 2005), backcrossed 6 generations onto the C57BL6 strain were housed according to the guidelines of the National Institutes of Health under an approved IACUC protocol at the University of North Carolina-Chapel Hill. Primary murine embryonic fibroblasts were isolated from day 13.5 embryos and grown in DMEM supplemented with 10% FCS. All MEF experiments were performed on cells within 2 cell passages of isolation from the mice.

Figure 2.1. siRNA mediated knockdown of DJ-1 and Affymetrix analysis. (**A**) End-point RT-PCR of H157 cells transfected with control siRNA (siCTL) or two different siRNA targeting DJ-1 (siDJ-1#1, siDJ-#2). The DJ-1 RT-PCR gel is presented as a negative image so bands can be more easily visualized. NTC is a non-template control. (**B**) Western blot analysis of siRNA transfected H157 cells demonstrating DJ-1 knockdown at the protein level. (**C**) Quantitative real-time PCR (Q-PCR) of DJ-1 mRNA following siRNA transfection. Relative mRNA quantitation is normalized to 18S rRNA expression. siDJ-1 #2 reduced DJ-1 expression to a greater degree than siDJ-1 #1, while transfection with either a scrambled non-specific oligomer siRNA or transfection reagent alone (siMock) did not affect DJ-1 expression.

Figure 2.1

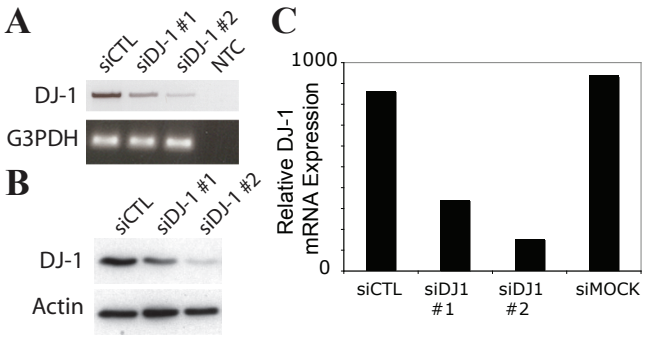

A
siCTL
siDJ-1 #1
siDJ-1 #2
NTC
DJ-1
G3PDH
B
siCTL
siDJ-1 #1
siDJ-1 #2
DJ-1
Actin
C
1000
0
Relative DJ-1 mRNA Expression
siCTL
siDJ1 #1
siDJ1 #2
siMOCK

Figure 2.2. Characterization of polyclonal rabbit anti-DJ-1 antibody. Western blots of human DJ-1 (hDJ-1), bacterially produced and purified recombinant human DJ-1 protein (rDJ-1), and mouse DJ-1 (mDJ-1). Left panel, human DJ-1 from H157 cell lysates transfected with either scrambled non-silencing siRNA (siCTL), or either of two non-overlapping siRNA oligomers specific to DJ-1 (siDJ-1 #1 & siDJ-1 #2) was immunoblotted with the anti-DJ-1 antibody. The antibody recognized a protein of the predicted mobility in siCTL treated cells, but not cells with the siDJ-1. The antibody also reacted with recombinant DJ-1. Right panel, mouse DJ-1 was also recognized by the anti-DJ-1 antibody. Mouse embryonic fibroblast (MEF) cell culture lysates from wild type DJ-$1^{+/+}$ cells and DJ-$1^{-/-}$ genetically ablated knockout mice were prepared. The presence of a single band at 24 kDa that is not present in DJ-$1^{-/-}$ cells shows the specificity of this antibody.

Figure 2.2

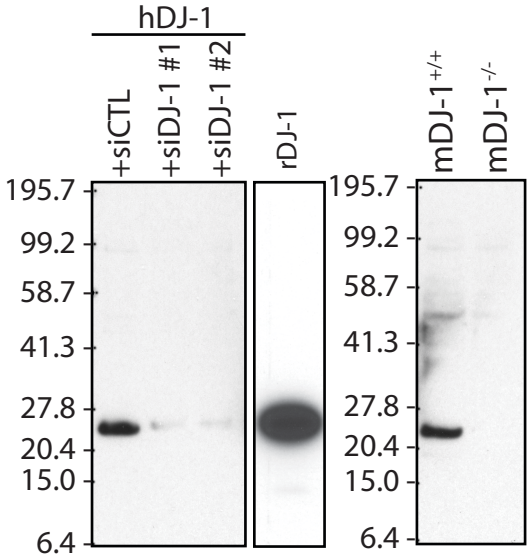

hDJ-1
+siCTL
+siDJ-1 #1
+siDJ-1 #2
rDJ-1
mDJ-1+/+
mDJ-1-/-
195.7
99.2
58.7
41.3
27.8
20.4
15.0
6.4
195.7
99.2
58.7
41.3
27.8
20.4
15.0
6.4

Figure 2.3. Summary of Affymetrix GeneChip analysis - Genes shown represent changes of greater than three (3) fold between siCTL and siDJ-1 transfected samples, where fluorescence in the present (P) state is greater than five hundred (500) in all samples. Green indicates decreased expression in normalized fluorescence while red indicates higher expression. Putative Nrf2 binding sequences within 1,000 bp upstream of the transcription start site are included to the right where present and identified using TF_search and a score of greater than eighty-five (85.0).

Figure 2.3

		Fold Change	Upstream ARE-like sequence
TOMM20	TRANSLOCASE OF OUTER MITOCHONDRIAL MEMBRANE 20	-5.02	
RRN3	RNA POLYMERASE I TRANSCRIPTION FACTOR	-4.81	**GGA**GG**CTGAG**GCA**CGA**GAAT
P24A/TMED2	TRANSMEMBRANE EMP24 TRANSPORT DOMAIN-CONTAINING PROT.	-4.34	**CGA**GG**CTGAG**GCA**CGA**GAAT
LAMP1	LYSOSOME-ASSOCIATED MEMBRANE PROTEIN 1	-4.33	
ABCC3	ATP-BINDING CASSETTE, SUBFAMILY C, MEMBER 3	-3.88	**ACT**CA**ATGAC**TCA**TCG**GCCC
CLNS1A	CHLORIDE CHANNEL, NUCLEOTIDE SENSITIVE, 1A	-3.79	**TTG**CG**CTGAG**TCA**GTT**CCTG
DJ-1	ONCOGENE DJ-1/PARK7	-3.69	**CGG**AC**GTGAC**GCA**GCG**TGAG
PSIP1	PC4- AND SFRS1-INTERACTING PROTEIN 1	-3.57	
OB-RG/LEPR	LEPTIN RELATED GENE/LEPTIN RECEPTOR	-3.49	
RAB14	RAS-ASSOCIATED PROTEIN	-3.38	
PIGB	PHOSPHATIDYLINOSITOL GLYCAN, CLASS B	-3.35	
NQO1	NAD(P)H DEHYDROGENASE, QUINONE 1	-3.32	**TCA**CA**GTGAC**TCA**GCA**GAAT
HRASLS3	HRAS-LIKE SUPPRESSOR 3	-3.31	
NOG1	NOGGIN HOMOLOG	-3.02	
GREM1	GREMLIN 1 HOMOLOG, CYSTEINE KNOT SUPERFAMILY	+4.00	
CALR	CALRETICULIN	+3.27	
CTGF	CONNECTIVE TISSUE GROWTH FACTOR	+3.10	**GCA**TC**CTGAG**TCA**CAC**GCGT

Figure 2.4. Expression profile of known Nrf2 regulated genes with respect to DJ-1 expression. The Nrf2 regulated gene network was generated using Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). A data set containing gene identifiers and corresponding expression values was uploaded in the application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. A Fold change cutoff of 1.4 fold was set to identify genes whose expression was significantly differentially regulated. This diagram is a graphical representation of the genes whose expression is controlled or altered by Nrf2. Gene products are represented as nodes, and the biological relationship (transcription or expression) between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation for changes >1.4 fold. Nodes are displayed using various shapes that represent the functional class of the gene product. The number under each gene name indicates the fold difference between DJ-1 expressing cells (3 arrays), and DJ-1 knockdown cells (2 arrays). Nodes without numbers were not present on the array or were excluded from analysis if its raw Affymetrix expression signal was less than 200, or if the gene was not changed more than 10% (1.10 fold) between the 3 DJ-1 expressing arrays and the 2 DJ-1 knockdown arrays. Among the genes identified, five were increased while two were decreased. The mechanism by which the presence of DJ-1 causes gene reduction is unclear.

Figure 2.4

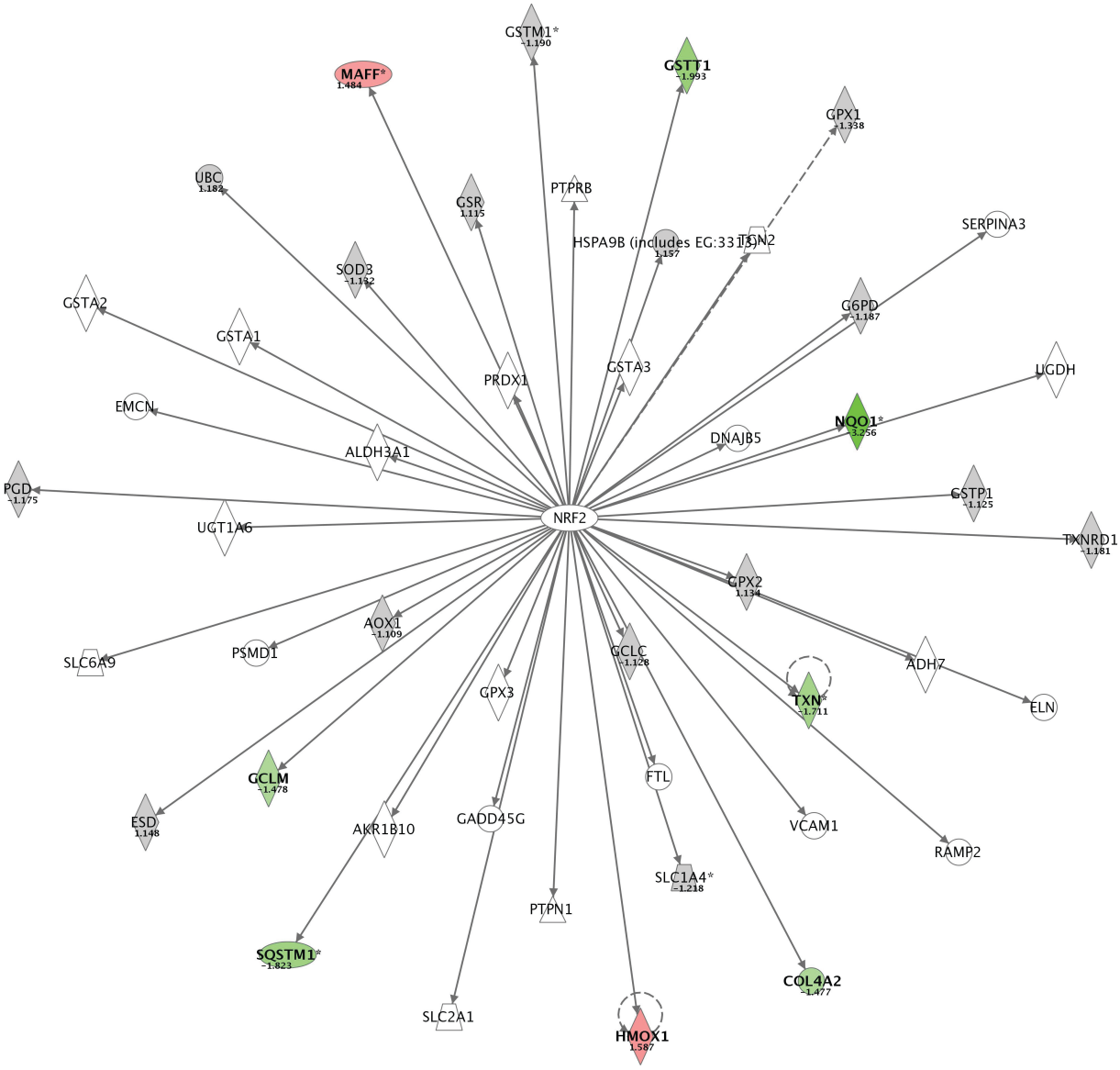

GSTM1*
-1.190
MAFF*
1.484
GSTT1
-1.993
GPX1
-1.338
UBC
1.182
GSR
1.115
PTPRB
HSPA9B (includes EG:3313)
1.157
TXN2
SERPINA3
SOD3
-1.132
GSTA2
G6PD
-1.187
GSTA1
GSTA3
PRDX1
UGDH
EMCN
NQO1*
3.256
DNAJB5
ALDH3A1
PGD
-1.175
GSTP1
-1.125
UGT1A6
NRF2
TXNRD1
-1.181
GPX2
1.134
AOX1
-1.109
SLC6A9
PSMD1
GCLC
-1.128
ADH7
GPX3
TXN*
-1.711
ELN
GCLM
-1.478
FTL
ESD
1.148
AKR1B10
GADD45G
VCAM1
RAMP2
SLC1A4*
-1.218
PTPN1
SQSTM1*
-1.823
COL4A2
-1.477
SLC2A1
HMOX1
1.587

Figure 2.5. DJ-1 is required for Nrf2 mediated transcription. (**A**) Real-time Q-PCR analysis of mRNA expression verifies that siDJ-1#2 reduced DJ-1 mRNA expression, as well as NQO1 mRNA expression. However, the mRNA of Nrf2, a master regulator of NQO1 expression, is unaffected by the loss of DJ-1. All samples were performed in triplicate and error bars indicate the standard error of the mean. (**B**) ARE-regulated luciferase reporter gene activity in Huh7 cells is reduced following siDJ-1 transfection. The firefly luciferase reporter construct is under the control of the NQO1 anti-oxidant response element (ARE) (Dhakshinamoorthy et al. 2000), which is responsive to Nrf2. Cells were then treated with 50 μM tBHQ or a DMSO vehicle control. Lysates were assayed for luciferase activity, and normalized to crude protein present in the extract. Flag-Nrf2 was transfected as a positive control. Samples with lowered DJ-1 expression contained lower levels of the ARE-regulated luciferase activity, and failed to increase luciferase activity following treatment with tBHQ. All samples were performed in triplicate and error bars indicate the standard error of the mean. (**C**) Luciferase activity expressed from a construct under the control of the constitutively active viral SV40 promoter was not affected by siDJ-1. (**D**) Luciferase activity expressed from two mammalian promoters was not affected by siDJ-1. Huh7 cells with siDJ-1 were transfected with luciferase reporter constructs under control of the NQO1 ARE [ARE], Glucocorticoid response element [GRE], or c-AMP response element [CRE]. Cultures were treated with the appropriate vehicle control or 50 μM tBHQ, 100 μM Dexamethasone (DEX), or 10 μM forskolin (FOR) respectively. Activation is presented as the percent induction of control oligomer (siCTL) transfected cells. All experiments in this figure were replicated at least three times.

Figure 2.5

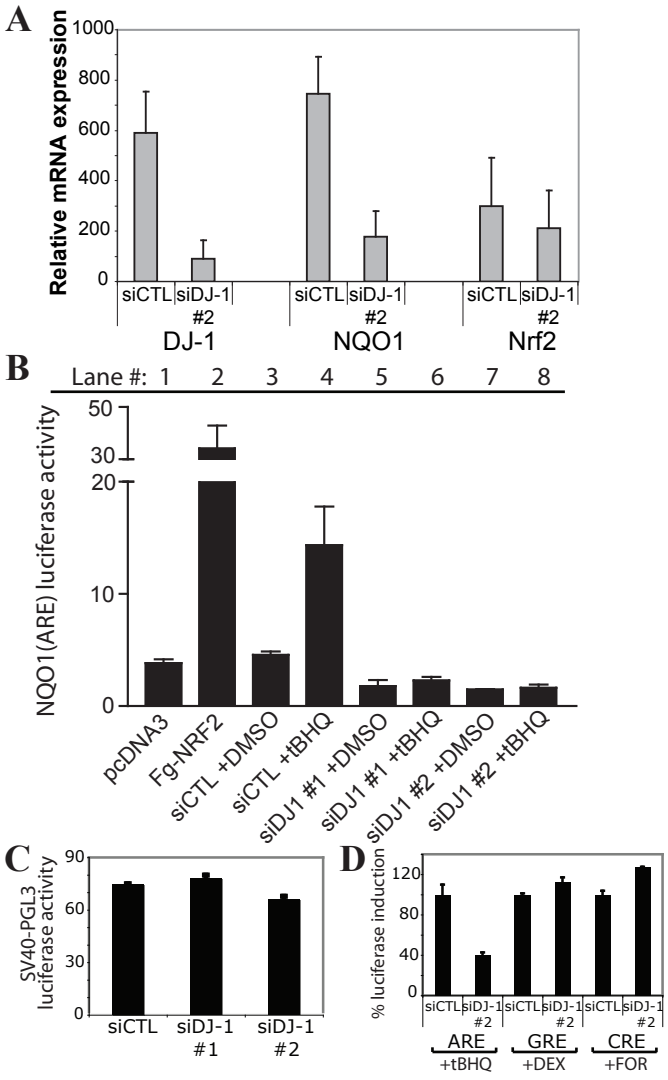

A
Relative mRNA expression
1000
800
600
400
200
0
siCTL
siDJ-1 #2
DJ-1
NQO1
Nrf2
B
Lane #: 1 2 3 4 5 6 7 8
NQO1(ARE) luciferase activity
50
30
20
10
0
pcDNA3
Fg-NRF2
siCTL +DMSO
siCTL +tBHQ
siDJ1 #1 +DMSO
siDJ1 #1 +tBHQ
siDJ1 #2 +DMSO
siDJ1 #2 +tBHQ
C
SV40-PGL3 luciferase activity
90
60
30
0
siCTL
siDJ-1 #1
siDJ-1 #2
D
% luciferase induction
120
80
40
0
siCTL
siDJ-1 #2
ARE +tBHQ
GRE +DEX
CRE +FOR

Figure 2.6. Chromatin immunoprecipitation (ChIP) of the NQO1 promoter. We assayed for the presence of DJ-1 bound to the NQO1 promoter by ChIP using antibodies specific for DJ-1 and Nrf2. In brief, we crosslinked cells using formaldehyde, lysed the cells, and sheared chromatin by sonication (as in Greer et. al.; Nat. Immunol. 2003 Nov;4(11):1074-82.). We then immunoprecipitated Nrf2 or DJ-1, digested the protein with proteinase K, and performed Sybr Green realtime Q-PCR with NQO1 promoter specific primers 5' CAGGACTCTCAGCCTTCCAA 3' and 5' TGGCACGAAATGGAGCAGAA 3' that amplify the region of the NQO1 promoter containing the ARE. Data were normalized to the promoter input into the immunoprecipitation. Values given are the quntity of promoter immunopreciptated as percent of input. Nrf2 was found bound to the NQO1 promoter, and its binding was enhanced by tBHQ treatment. However, DJ-1 ChIP was not able to enrich for the NQO1 promoter sequence in either treatment, indicating that the association of DJ-1 with this promoter is not detected.

Figure 2.6

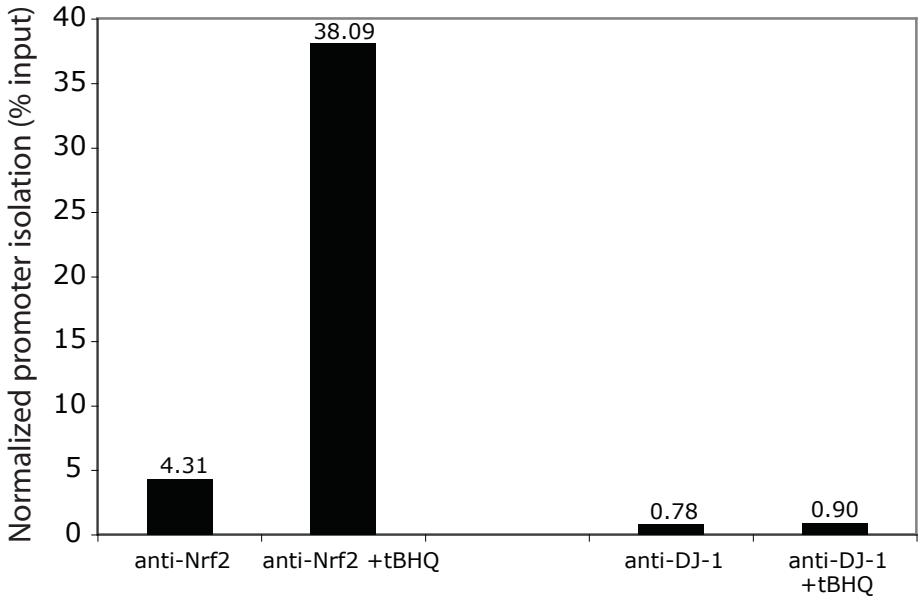

Normalized promoter isolation (% input)
40
35
30
25
20
15
10
5
0
38.09
4.31
0.78
0.90
anti-Nrf2
anti-Nrf2 +tBHQ
anti-DJ-1
anti-DJ-1 +tBHQ

Figure 2.7. DJ-1 does not alter Keap1 mRNA expression. Keap1 mRNA expression was measured using SYBR Green RealTime qPCR with the primers 5' GGCGAATGATCACAGCAATG 3', and 5' GCTGGTCCTGACCATCATAG 3', and expression was normalized to 18S rRNA expression. In the main text, the data indicate that siDJ-1 decreased NQO1 transcription and reduced Nrf2 protein stability. However, as this realtime qPCR analysis shows, DJ-1's effect is not mediated by differences in Keap1 mRNA expression. All samples were performed in triplicate and error bars indicate the standard error of the mean.

Figure 2.7:

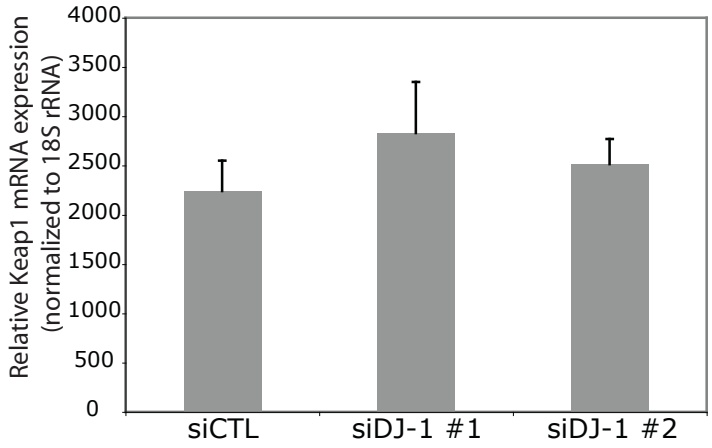

Relative Keap1 mRNA expression
(normalized to 18S rRNA)
4000
3500
3000
2500
2000
1500
1000
500
0
siCTL
siDJ-1 #1
siDJ-1 #2

Figure 2.8. DJ-1 is required for Nrf2 protein stability. (**A**) Western blot analysis of Nrf-2, DJ-1 and control proteins in Huh7 cell lysates following siRNA knockdown of DJ-1. (**B**) Time-course of protein expression following cyclohexamide (CHX) treatment. Western blot analysis confirms the presence of Nrf2 at times following CHX treatment in control samples. Actin is used as an unaffected control. (**C**) *In cellulo* assay of Nrf2 ubiquitinylation. Nrf2 and covalently bound ubiquitin were immunoprecipitated from Huh7 extracts, and analyzed by SDS-PAGE western blot analysis. (**D**) Nrf2/Keap1 co-immunoprecipitation in the presence of DJ-1. V5 epitope tagged Keap1 was expressed in Huh7 cells with and without over-expressed flag-DJ-1. Immunoprecipitation using anti-V5 antibody co-immunoprecipitated endogenous Nrf2 protein and conversely, immunoprecipitation of endogenous Nrf2 co-isolated V5-Keap1. H.C. denotes a cross-reacting band of IgG heavy chain present from the immunoprecipitating antibody. These data are representative of at least three independent experiments.

Figure 2.8

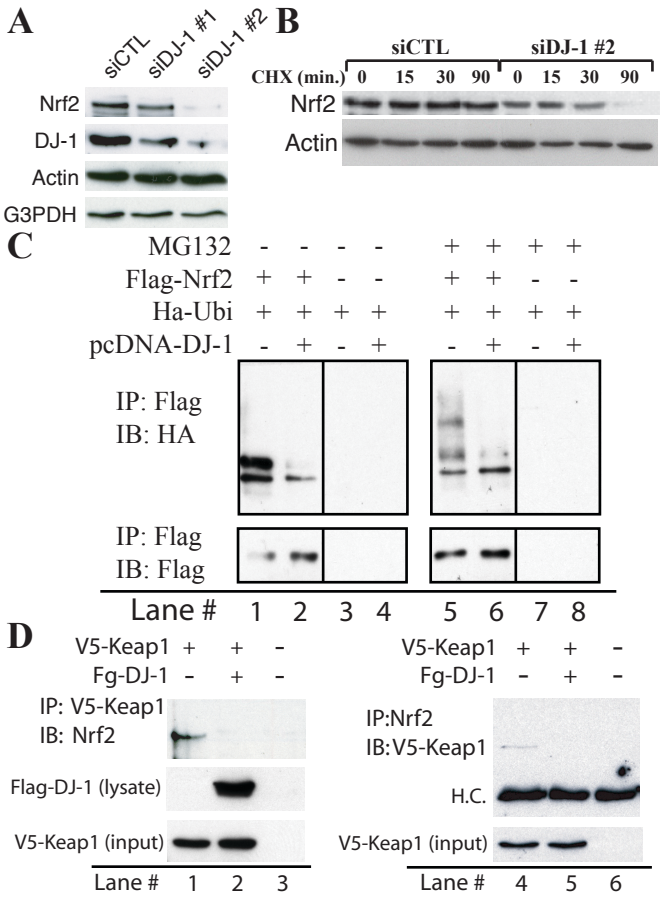

A
siCTL
siDJ-1 #1
siDJ-1 #2
Nrf2
DJ-1
Actin
G3PDH
B
siCTL
siDJ-1 #2
CHX (min.) 0 15 30 90 0 15 30 90
Nrf2
Actin
C
MG132 - - - - + + + +
Flag-Nrf2 + + - - + + - -
Ha-Ubi + + + + + + + +
pcDNA-DJ-1 - + - + - + - +
IP: Flag
IB: HA
IP: Flag
IB: Flag
Lane # 1 2 3 4 5 6 7 8
D
V5-Keap1 + + -
Fg-DJ-1 - + -
IP: V5-Keap1
IB: Nrf2
Flag-DJ-1 (lysate)
V5-Keap1 (input)
Lane # 1 2 3
V5-Keap1 + + -
Fg-DJ-1 - + -
IP:Nrf2
IB:V5-Keap1
H.C.
V5-Keap1 (input)
Lane # 4 5 6

Figure 2.9. Nrf2 pathway proteins did not co-immunoprecipitate with DJ-1. Using HEK293T cells, we examined DJ-1 protein-protein interactions with Nrf2 and proteins that affect Nrf2 protein stability. V5-Keap1 expression construct was used due to the poor quality of commercially available Keap1 antibodies. (A) In contrast to the co-immunoprecipitation of V5-Keap1 and Nrf2, co-immunoprecipitation of DJ-1 with Nrf2 was not detected . Western blot analysis of DJ-1 immunoprecipitation eluates with DJ-1 antibody shows that the anti-DJ-1 antibody is capable of immunoprecipitation. For this DJ-1 blot, TruBlot anti-Rabbit-HRP (Insight Biotech) was used as a secondary antibody to eliminate any blotting of IgG light-chain that would complicate DJ-1 IP/IB blots. (B) Similar to the previous panel, immunoprecipitation of DJ-1 with V5-Keap1 was not detected, while immunoprecipitation of Nrf2 using the H-300 antibody (Santa Cruz Biotech) does isolate V5-Keap1. (C) Keap1 targets Nrf2 for protein degradation via Cullin-3 dependent ubiquitination; therefore we also assayed for DJ-1 protein interaction with Cullin-3. Using rabbit polyclonal anti-Cullin-3 (from Furukawa et. al. Nat. Cell Bio. 2003 Nov;5(11):1001-7) we immunoprecipitated Cullin-3 containing protein complexes and blotted for Flag-DJ-1. Flag-DJ-1 was used to eliminate background caused by the high expression of endogenous DJ-1. H_2O_2 indicates overnight treatment with 300 μM hydrogen peroxide to model oxidative stress. Flag-DJ-1 was not co-immunoprecipitated with Cullin-3 in these cells.

Figure 2.9

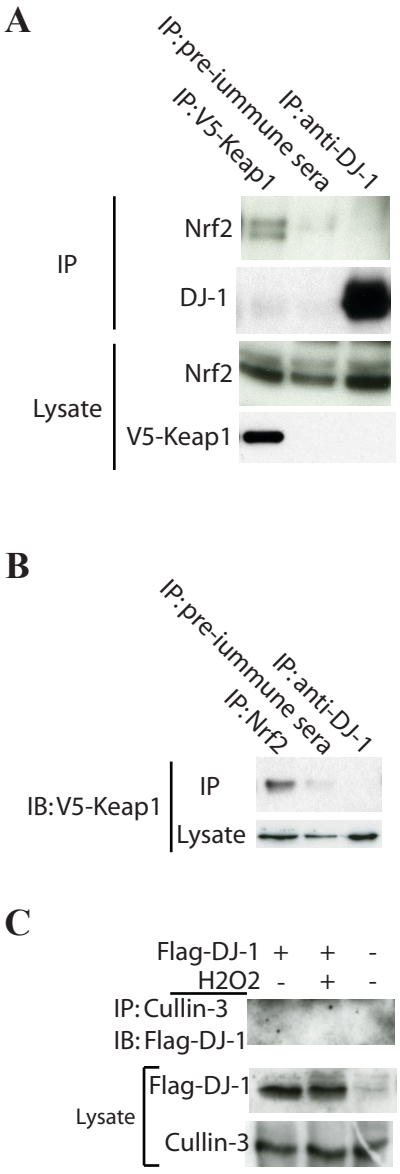

A
IP:pre-iummune sera
IP:anti-DJ-1
IP:V5-Keap1
IP
Nrf2
DJ-1
Lysate
Nrf2
V5-Keap1
B
IP:pre-iummune sera
IP:anti-DJ-1
IP:Nrf2
IB:V5-Keap1
IP
Lysate
C
Flag-DJ-1 + + -
H2O2 - + -
IP: Cullin-3
IB: Flag-DJ-1
Lysate
Flag-DJ-1
Cullin-3

Figure 2.10. DJ-1 is required for Nrf2 function in mouse embryonic fibroblasts. (**A**) Western blot analysis of mNrf2 protein expression in primary mouse embryonic fibroblasts (MEFs) derived from DJ-1 gene deletion mice and wild-type littermates. Cultures were treated with tBHQ at 0, 50, & 100 μM. 'm' designates that the genes are murine. (**B**) Western blot analysis of mNrf2 cultures transfected with either pcDNA or Flag-DJ-1 and treated with 50 μM tBHQ or vehicle control. (**C**) ARE-Luciferase activity in DJ-$1^{+/+}$ and DJ-$1^{-/-}$ MEF cultures. Luciferase is under control of the ARE from the human NQO1 gene promoter (left panel), while SV40-Luciferase is under control of the constitutively active viral SV40 promoter (right panel). Luciferase activity is normalized to protein concentration in the extract. (**D**) Real-time quantitative PCR of Nrf2-mediated target gene expression in primary MEFs. NQO1 is NAD(P)H quinone oxidoreductase I. GCLM is glutathione cysteine ligase, modifier subunit. Data is presented as fold induction following treatment with tBHQ compared with vehicle control. All mRNA measurements are normalized to mouse G3PDH expression. All samples were performed in triplicate and error bars indicate the standard error of the mean. These are representative data of at least 3 independent experiments.

Figure 2.10

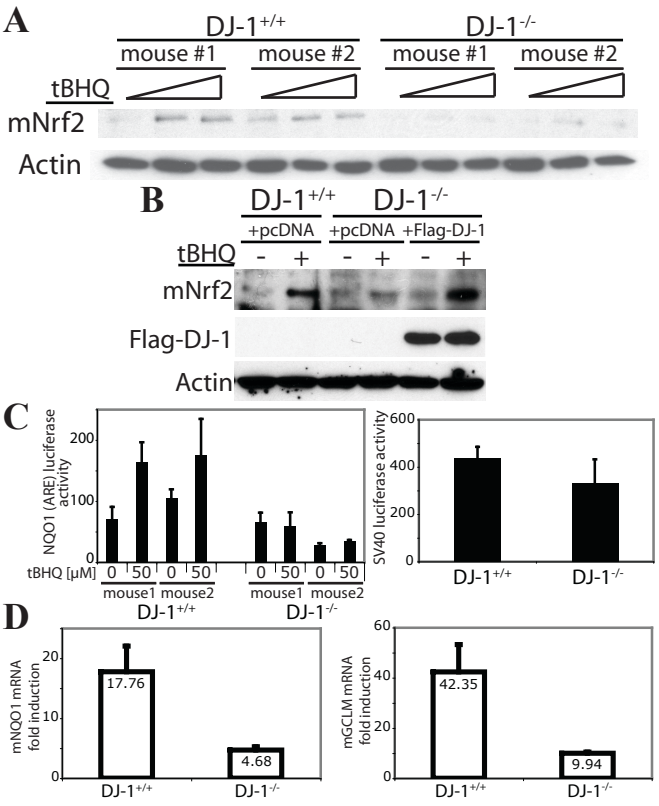
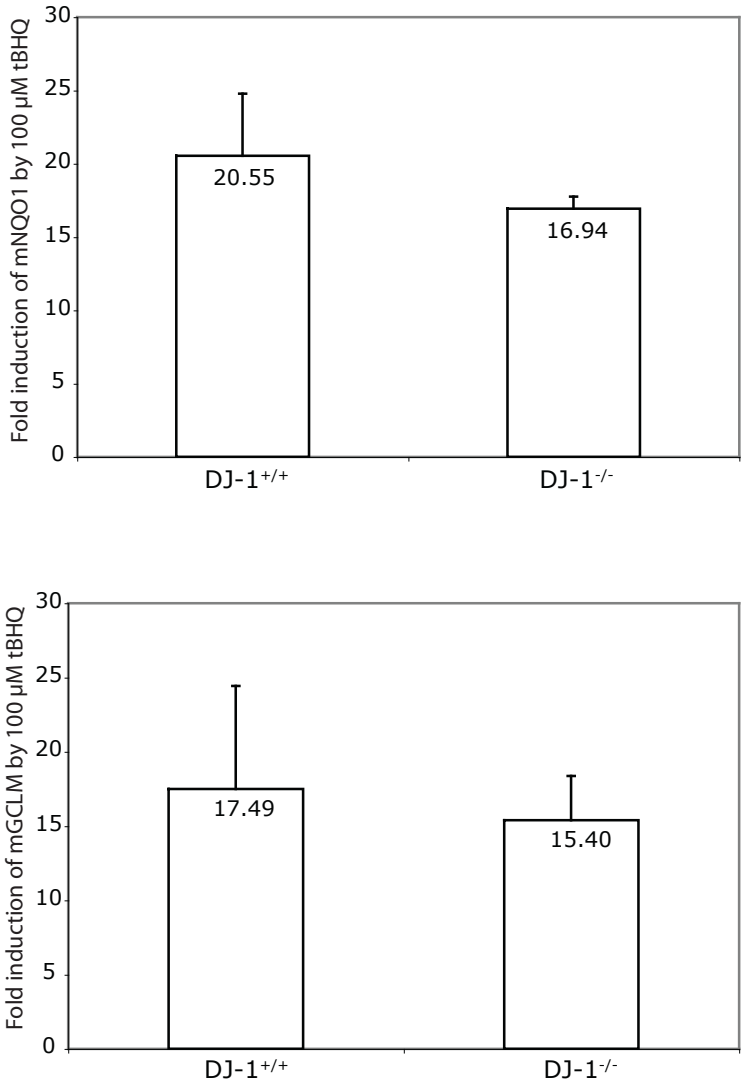

A
DJ-1+/+
DJ-1-/-
mouse #1
mouse #2
mouse #1
mouse #2
tBHQ
mNrf2
Actin
B
DJ-1+/+
DJ-1-/-
+pcDNA
+pcDNA
+Flag-DJ-1
tBHQ
- + - + - +
mNrf2
Flag-DJ-1
Actin
C
NQO1 (ARE) luciferase activity
200
100
0
tBHQ [μM]
0 50 0 50 0 50 0 50
mouse1 mouse2 mouse1 mouse2
SV40 luciferase activity
600
400
200
0
DJ-1+/+
DJ-1-/-
D
DJ-1+/+
DJ-1-/-
mNQO1 mRNA fold induction
20
10
0
17.76
4.68
DJ-1+/+
DJ-1-/-
mGCLM mRNA fold induction
60
40
20
0
42.35
9.94
DJ-1+/+
DJ-1-/-

Figure 2.11. High dose tBHQ induction of mouse NQO1 and GCLM genes are less dependent on DJ-1. The main text shows that MEFs lacking DJ-1 do not induce mNQO1 or mGCLM expression to the same extent as wild-type MEFs following tBHQ treatment. However, at higher doses, exceeding 100 μM tBHQ, induction of Nrf2 regulated genes approaches levels similar to DJ-$1^{+/+}$ cells. All samples were performed in triplicate in each of two mice and error bars indicate the standard error of the mean.

Figure 2.11


Fold induction of mNQO1 by 100 µM tBHQ
30
25
20
15
10
5
0
20.55
16.94
DJ-1+/+
DJ-1-/-
Fold induction of mGCLM by 100 µM tBHQ
30
25
20
15
10
5
0
17.49
15.40
DJ-1+/+
DJ-1-/-

CHAPTER III:

Protein interaction analysis and characterization of the cancer and Parkinson's disease associated protein, DJ-1

3.1 ABSTRACT:

DJ-1, a Parkinson's disease and cancer associated protein, protects cells during periods of stress prolonging survival. However, the mechanism of this cytoprotection remains unclear. It has been suggested that DJ-1 exists as a component of large molecular weight protein complexes in the brain and that DJ-1 function is mediated through its protein binding because DJ-1 has no known enzymatic function. DJ-1 protein-protein interactions are not as well characterized in peripherally derived cells or transformed cells. This study shows that DJ-1 in such cells exists in an unbound state under normal conditions, but following oxidative stress forms higher order protein complexes. These oxidation sensitive complexes require the cysteine at position 106 in DJ-1, which is shown to be directly oxidized under physiological oxidative potentials. Furthermore, we characterize the functional effect of DJ-1 interaction with Cezanne, an A20 family deubiquitinating enzyme. DJ-1 inhibits Cezanne deubiquitinase activity *in vitro* and reverses the inhibitory effect of Cezanne on NF-κB following TNFα treatment. We show that DJ-1 also inhibits the deubiquitinating enzyme Isopeptidase-T *in vitro,* suggesting a wider role for DJ-1 in regulating deubiquitinating enzymes. Finally, A20, the prototypic member of the deubiquitinating enzyme family including Cezanne, down regulates the antioxidant transcription factor Nrf2, which we have previously shown to be dependent on DJ-1. These results suggest that DJ-1 responds to oxidative stress by modifying cellular signaling cascades via deubiquitinating enzymes leading to cell survival.

3.2 INTRODUCTION:

The *dj-1/park7* gene encodes a multifunctional protein that promotes cell survival and protects cells from toxic insults including oxidative stress (MacKeigan et al. 2003; Martinat et al. 2004; Taira et al. 2004; Meulener et al. 2005; Zhou et al. 2005; Clements et al. 2006). Initially described for its ability to transform cells in culture (Nagakubo et al. 1997), DJ-1 has since been identified as a biomarker for several cancer types (MacKeigan et al. 2003; Hod 2004), and DJ-1 expression is associated with poor cancer prognosis (Kim et al. 2005). Furthermore, functional loss of DJ-1 is one of the few known genetic causes of Parkinson's disease (Bonifati et al. 2003), a common neurodegenerative disorder characterized by loss of dopaminergic neurons in the brain and subsequent motor deficits.

While the effects of DJ-1 on cell survival are prominent and well reported, and DJ-1 dysfunction is clearly associated with disease, the mechanisms of DJ-1 function remain obscure. We have previously shown that DJ-1 is required for intact antioxidant transcription mediated by Nrf2 (Clements et al. 2006). Although we showed a profound functional effect of DJ-1 on Nrf2 protein stability and activity, we were unable to find DJ-1 physically interacting in complex with several known proteins that regulate Nrf2 activity including, Keap1, Cullin-3, or Nrf2 itself (Itoh et al. 1999; Kobayashi et al. 2004; Zhang et al. 2004; Furukawa et al. 2005).

DJ-1 is a member of the PfpI/ThiJ protein family. While this family shares significant sequence and structural homology, its members provide various and sundry functions including protease, kinase, aminotransferase, and protein chaperone

functionality (Loewen et al. 1993; Halio et al. 1996; Mizote et al. 1999; Du et al. 2000; Sastry et al. 2002). Previous structural and functional studies have failed to show any enzymatic activity of DJ-1 (Honbou et al. 2003; Huai et al. 2003; Lee et al. 2003; Wilson et al. 2003); however, DJ-1 has been proposed to exist in a large molecular weight protein complex in the brain (Baulac et al. 2004; Meulener et al. 2005) and has been shown to prevent protein aggregation *in vitro* (Lee et al. 2003; Shendelman et al. 2004). Therefore, we propose that DJ-1 function is dependent on protein-protein interactions in the cell and identification of DJ-1 interacting proteins will further elucidate the direct functional effects of DJ-1.

Several proteins have been proposed to interact with DJ-1. These studies have been performed with respect to neurological and testicular biology, both of which have been suggested to be sensitive to DJ-1 malfunction (Wagenfeld et al. 1998; Taira et al. 2001; Takahashi et al. 2001; Niki et al. 2003; Kim et al. 2005). Notably, DJ-1 is reported to interact with Parkin (Park2) (Moore et al. 2005) and Pias2α proteins (Takahashi et al. 2001), which function as ubiquitin and SUMO (ubiquitin like modification) E3 ligases respectively. Other reported DJ-1 interactions include cell survival signaling proteins (Karunakaran et al. 2007) and proteins regulating gene expression (Hod et al. 1999; Niki et al. 2003; Taira et al. 2004). DJ-1 interactions in cancer/transformed cells have not been extensively studied to date. Additionally, while DJ-1 has been shown to be directly oxidized under physiological oxidative potentials (Mitsumoto et al. 2001; Mitsumoto et al. 2001), and this oxidation has been mapped to a specific cysteine residue within DJ-1 (Kinumi et al. 2004), the effects of DJ-1 oxidation on protein interactions have not been studied.

In the current study we use an unbiased approach to identify and functionally characterize DJ-1 interacting proteins in transformed human epithelial cells. In a basal state, DJ-1 exists in a very small molecular weight complex consistent with monomeric/dimeric unbound DJ-1. However, when DJ-1 is oxidized it becomes more promiscuous in its binding. We have identified several previously unknown DJ-1 interacting proteins that bind DJ-1 after cysteine 106 is oxidized. We functionally characterize the interaction of DJ-1 with Cezanne, a deubiquitinating enzyme that downregulates NF-κB activation following TNFα stimulation (Evans et al. 2001; Evans et al. 2003). We show that DJ-1 directly inhibits Cezanne catalyzed deubiquitination *in vitro*, and that DJ-1 positively regulates NF-κB activity. Suggesting a broader role for DJ-1 in regulating deubiquitinating enzymes, DJ-1 is able to inhibit Isopeptidase-T cleavage of ubiquitin *in vitro*. This led us to examine the effect of deubiquitinating enzyme activity on Nrf2, the master regulator of antioxidant transcription, which we have previously shown to depend on DJ-1. We find that A20, a deubiquitinating enzyme closely related to Cezanne, functions to inhibit Nrf2, linking A20 to the regulation of a transcription factor that is important for modulating oxidative-stress responses.

3.3 RESULTS:

Mass spectrometric identification of oxidation-specific DJ-1 interacting proteins:

To identify proteins that interact with DJ-1, we adopted an unbiased proteome-wide survey of DJ-1 containing complexes. Due to the high level of DJ-1 express by almost all cells examined, we used mouse embryonic fibroblasts (MEFs) isolated from DJ-1 knockout mice, and restored DJ-1 expression by transfection with plasmids encoding flag epitope tagged wild type DJ-1 or DJ-1 whose cysteine at position 106 was mutated to an alanine (C106A). Previous functional and structural studies of DJ-1 have shown that the cysteine at position 106 in the DJ-1 polypeptide could be oxidized to a sulfinic acid at physiologic oxidative potentials (Mitsumoto et al. 2001; Mitsumoto et al. 2001; Kinumi et al. 2004) and that this residue is maintained in an orientation with strained bonds to be exposed for binding (Honbou et al. 2003; Huai et al. 2003; Lee et al. 2003; Wilson et al. 2003). At low dose hydrogen peroxide, C106 oxidation is responsible for a shift in the pI of DJ-1; therefore, we wanted to test the effect of C106 oxidation in mediating oxidation sensitive DJ-1 protein-protein interactions. Additionally a negative control was analyzed in parellel using MEFs that were transfected with a control plasmid containing no insert (pcDNA).

Cells were treated with hydrogen peroxide or media vehicle, and DJ-1 containing complexes were immunoprecipitated using M2 anti-Flag agarose (Sigma). Peptide mixtures were analyzed by quadrupole-time of flight (Q-TOF) LC/MS/MS to obtain mass spectra, and ion mass series were searched against the existing databases of known

proteins using the Mascot search engine (Matrix Science) to identify the proteins in solution that were isolated in each sample. Peptides corresponding to trypsin, immunoglobulins, or peptides isolated by the anti-Flag immunoprecipitation of the empty vector control were removed from analysis. These proteins consisted of cytoskeletal cross-contaminants (such as keratins).

In wild type DJ-1 containing cultures maintained at basal non-oxidative conditions, we did not identify any specific protein other than DJ-1. However, oxidative treatment of wild type DJ-1 cultures induced DJ-1 protein complex formation, and four proteins were identified as putative DJ-1 interactions in MEFs. These proteins were Bardet-Biedl Syndrome-1 (BBS1), the A20 family deubquitinating enzyme Cezanne (Za20d1), an organic anion transporter protein (OATP-r), and a putative sugar kinase (NadK) (Figure 3.1A).

These interactions were only detected with wild type DJ-1 in the presence of hydrogen peroxide. When cultures containing the DJ-1 C106A mutant protein were analyzed, we did not detect any specific interacting proteins regardless of oxidative treatment. This implicates cysteine-106 oxidation to sulfinic acid as a critical step in regulating the interaction of DJ-1 with other proteins.

DJ-1 co-immunoprecipitates with BBS1 and Cezanne:

To verify the interactions identified in the MEFs by mass spectrometry, we isolated the cDNA encoding putative DJ-1 interacting proteins and co-expressed them along with DJ-1 in 293T cells. Following oxidation with hydrogen peroxide, we immunoprecipitated the putative interacting proteins, which are V5 epitope tagged, and

western blotted for the co-expressed DJ-1 protein. We found that DJ-1 co-immunoprecipitated with both human Cezanne and mouse BBS1 proteins (Figure 3.1B). As a control, cells containing empty vector and cells containing an unrelated V5-epitope tagged protein, WDR5, were immunoprecipitated and co-expressed DJ-1 was not bound.

Under culture conditions DJ-1 is not present in large molecular weight complexes

Given that DJ-1 has been reported to form high molecular weight complexes in the brain, we analyzed peripherally derived transformed epithelial cells for DJ-1 protein complex size. Using gel filtration chromatography, we measured the size of DJ-1 containing protein complexes in HT-29 cell lysates. Using a SuperDex75 gel filtration column to fractionate small molecular weight complexes, we injected BSA (66 kD), recombinant DJ-1 (monomer 24 kD, dimer 48 kD), and lysate from HT-29 cells. We then immunoblotted the isolated fractions from HT-29 lystates for the presence of DJ-1. All of the DJ-1 in the lysate was able to enter the gel matrix, showing that DJ-1 complexes are less than the 100 kD exclusion limit of the column. DJ-1 was eluted from the column in the same fractions as purified recombinant DJ-1 (fractions 19-22). This shows that a significant fraction of DJ-1 remains unbound under non-oxidative conditions (Figure 3.2).

DJ-1 and the putative interacting proteins are cytosolic

The cellular localization of proteins can control their biological activity and protein-protein interactions. To determine the cellular localization of DJ-1 and its putative protein interacting partners, we expressed epitope tagged proteins in Huh7 liver

hepatoma cells. These cells were chosen because they are large, have easily visible morphology, and express transfected plasmids at modest levels, minimizing the localization effects of over-expression. Cells were then fixed and stained with fluorescent antibodies against the protein epitopes and imaged by confocal microscopy. DJ-1 shows a diffuse cytoplasmic staining pattern. The putative interacting proteins also show a cytoplasmic localization with NadK and Cezanne most congruous with DJ-1 localization. BBS1 is localized both to the cytoplasm and the nucleus. These results suggest that NadK, BBS1, and Cezanne are present in the same cellular areas as DJ-1 and could bind DJ-1 in the cytosol (Figure 3.3).

DJ-1 activates NF-κB reporter gene transcription

Given the well studied link between ubiquitination and Parkinson's disease, as well as the effects of DJ-1 on ubiquitin and ubiquitin-like protein conjugation reported by our group and others, we set out to determine the effect of DJ-1 on Cezanne function. Since DJ-1 is found in an oxidation dependent complex with Cezanne, and Cezanne functions to down regulate NF-κB activation following TNFα treatment (Evans et al. 2001; Evans et al. 2003), we used NF-κB reporter gene assays to determine the effect of DJ-1 on NF-κB activity with respect to Cezanne.

Using 293T cells containing a reporter gene construct that encodes the firefly luciferase gene under control of a NF-κB responsive promoter, we activated NF-κB signaling by TNFα treatment (Figure 3.4A). Expression of Cezanne inhibited NF-κB activation following TNFα. Co-expression of DJ-1 with Cezanne reversed that effect,

returning NF-κB driven luciferase expression to the same level as cells without Cezanne over-expression. This suggests that DJ-1 inhibits Cezanne function and cumulatively acts as a positive regulator of NF-κB mediated gene expression.

Expression of the prototypic NF-κB inhibitor protein A20 strongly down regulated NF-κB driven luciferase expression, and while co-expression of DJ-1 did increase NF-κB transcription somewhat, the effect was moderate. As a control, these experiments were repeated in cells containing a luciferase reporter construct driven by the cAMP-responsive element (CRE). In these control cultures, expression of DJ-1, A20, and Cezanne had no effect on CRE activiation by Forskalin treatment (Figure 3.4B)

DJ-1 inhibits Cezanne mediated breakdown of poly-ubiquitin chains:

Characterized by its similarity to the deubiquitinating enzyme A20, Cezanne has been shown to down regulate NF-κB mediated transcription following TNFα treatment by catalyzing the breakdown of poly-ubiquitin chains. (Evans et al. 2001; Evans et al. 2003). To study any direct effect of DJ-1 on deubiquitinating enzyme function, we generated recombinant Cezanne expressed in bacteria and purified by metal coordination chromatagrophy (Figure 3.5 A,B,C). Enrichment of the Cezanne protein over a nickel column yields a band corresponding to full length Cezanne, and several smaller molecular weight protein bands. The majority of these bands are immunoreactive against the V5 epitope contained at the carboxy-terminus of Cezanne indicating that they are likely N-terminal truncated proteins. A Cezanne antibody raised against an internal peptide also

recognized full-length Cezanne and the smaller molecular weight bands. Therefore the Cezanne protein preparation contains full length Cezanne and its degradation products.

Previous work has shown that Cezanne catalyzes the breakdown of poly-ubiquitin chains. To characterize the effects of DJ-1 on Cezanne catalyzed breakdown of ubiquitin chains, we reacted recombinant Cezanne (rCez) with branched ubiquitin chains ranging in size from two to seven ubiquitin repeats per molecule. Incubation with rCez or the positive control, Isopeptidase-T (IsoT), cleaved poly-ubiquitin chains (Evans et al. 2001; Evans et al. 2003). Traditionally thought of as a degradative signal, the conjugation of ubiquitin can also act as an activating signal for protein function. Generally, the conjugation of ubiquitin monomers via the lysine residue at position 48 (K48) signals the proteosomal degradation of a protein (Pickart 1997), while activating ubiquitination is often linked through lysine at position 63 (K63) (Deng et al. 2000). Incubation of Isopeptidase-T with either branched ubiquitin moiety causes efficient breakdown of the ubiquitin branches to monomeric ubiquitin. (Figure 3.5 D, E,) Incubation of K48 linked ubiquitin chains with rCez causes degradation of the ubiquitin branches to a form no longer recognized by the ubiquitin antibody. Treatment of K63 branched ubiquitin on the other hand is broken down by rCez to a pentamer (5xUb) and no further (Figure 3.5 E). Titration of rDJ-1 into these reactions causes a dose dependent inhibition of ubiquitin cleavage by rCez. This effect is most striking on K48-branched ubiquitin chains because Cezanne preferentially degrades these chains. These findings suggest that DJ-1 prevents Cez from degrading poly-ubiquitin chains.

DJ-1 inhibits Isopeptidase-T mediated breakdown of ubiquitin mimicking peptide:

Because DJ-1 is able to inhibit Cezanne function in vitro, we wanted to test the effect of DJ-1 on deubiquitinating enzymes more generally. Isopeptidase-T is a prototypic deubiquitinating enzyme that targets the carboxy terminus of ubiquitin, cleaving branched ubiquitin chains to monomeric ubiquitin. The activity of Isopeptidase-T can be quantitatively and kinetically measured using a peptide mimetic of ubiquitin (Stein et al. 1995). A peptide sequence from the C-terminus of ubiquitin bound to the fluorochrome AMC quenches the fluorescent signal, and its cleavage releases AMC, which can then be measured by fluorescence (Figure 3.5 F). Isopeptidase-T was able to cleave the peptide mimetic efficiently, while the peptide alone remained stable throughout the course of the experiments. Interestingly, titration of rDJ-1 inhibited Isopeptidase-T mediated breakdown of the AMC-peptide. At a 1:1 molecular ratio of DJ-1 to Isopeptidase-T, peptide breakdown was slightly inhibited; however, at a 4:1 DJ-1 to IsoT molecular ratio Isopeptidase-T function was decreased by greater than 50%. (Figure 3.5F)

The deubiquitinating enzyme, A20, negatively regulates Nrf2 transcription

Previously, our lab reported that DJ-1 is required for intact antioxidant responses mediated by the transcriptional master regulator, Nrf2. Our finding that DJ-1 inhibits both Cezanne and Isopeptidase-T in vitro, led us to consider the general role of deubiquitinating enzymes in Nrf2 regulation.

Using 293T cells that contain a reporter gene plasmid that encodes the firefly luciferase gene under control of the antioxidant responsive element (ARE), which is

bound and activated by Nrf2, we tested the effect of the deubiquitinating enzymes Cezanne and A20. (Figure 3.6) Nrf2 is activated under periods of toxic and oxidative stresses. To minimize the effects of global oxidation and toxic insults we activated Nrf2 using the non-toxic food preservative ter-butylhydroquinone (tBHQ). tBHQ treatment activates Nrf2 and, to date, has not been reported to affect other pathways. Treatment of the reporter-gene containing cell culture induced a robust Nrf2 response, the addition of DJ-1 produced a modest increase in Nrf2 activation in these cells, which contain relatively high endogenous DJ-1 protein expression. Transfection with the deubiquitinating enzyme, Cezanne, did not affect Nrf2 mediated reporter gene transcription. However, transfection with another deubiquitinating enzyme from the same family, A20, drastically reduced Nrf2 activated luciferase activity. Similar to NF-κB mediated gene expression; the co-expression of DJ-1 with A20 increased Nrf2 mediated reporter gene expression only moderately.

A20 decreases Nrf2 protein levels independent of Nrf2 ubiquitination

Nrf2 activity is mediated to large extent by protein stabilization. In the absence of oxidative stress, Nrf2 is bound to its cytosolic inhibitor protein Keap1. Keap1 targets Nrf2 for ubiquitination and subsequent proteosomal degradation (Kobayashi et al. 2004; Zhang et al. 2004; Furukawa et al. 2005). During periods of oxidative stress Nrf2 is released from Keap1, stabilizing Nrf2 and allowing antioxidant gene transcription to proceed (Wild et al. 1999; Chen et al. 2004, Itoh, 1997 #18). Our finding that A20

potently inhibited Nrf2 mediated reporter gene transcription led us to study the effects of A20 on Nrf2 protein stability.

293T cells treated with tBHQ stabilize Nrf2 protein leading to its accumulation over time (Figure 3.7A). Supra-physiologic expression of A20 leads to decreased levels of Nrf2 protein in these cells following tBHQ treatment. Consistent with our reporter gene assays, the introduction of DJ-1 does not restore Nrf2 protein levels to normal.

Given the ubiquitin-mediated degradation of Nrf2 by Keap1, and the ubiquitin editing functions of A20, we examined the effects of A20 on Nrf2 ubiquitination. By expressing Nrf2 in 293T cells and then immunoprecipitating denatured Nrf2, we detected ubiquitin covalently linked to Nrf2. Treatment of these cells with the proteosome inhibitor, MG132, causes the accumulation of ubiquitinated proteins that would otherwise be degraded by the proteosome. Therefore the detection of ubiquitinated Nrf2 represents the levels of Nrf2 protein that were ubiquitinated over the course of the MG132 treatment.

Over-expression of A20, which leads to decreased levels of Nrf2 protein, did not cause an increase in Nrf2 ubiquitination. In fact A20 caused the opposite: we were unable to detect ubiquitinated Nrf2 in cultures in which A20 was expressed. This suggests a role for A20 in Nrf2 regulation independent of ubiquitin mediated degradation, or Nrf2 effects that occur prior to the MG132 treatment. Again, co-expression of A20 with DJ-1 does not restore Nrf2 expression to normal levels suggesting that DJ-1's effect on A20 is moderate.

Control cultures lacking V5-epitope tagged Nrf2 showed that ubiquitination measured in this assay are specific. Also, a control in which Nrf2 ubiquitination was measured in the presence of over-expressed DJ-1 showed a decrease in Nrf2 ubiquitination consistent with the stabilizing effect of DJ-1 on Nrf2, and with our previous publication (Clements et al. 2006).

3.4 DISCUSSION

While previous reports of DJ-1 protein interactions in the context of the central nervous system have suggested a rich and plentiful network of DJ-1 interacting proteins (Baulac et al. 2004; Meulener et al. 2005), we find a different scenario in peripheral epithelial cells. While DJ-1 is expressed at very high levels in all of the cells we examined, the overwhelming majority of DJ-1 remains unbound from proteins in basal conditions of cell culture, consistent with a monomeric or dimeric state of DJ-1. However, following oxidation, DJ-1 binds several proteins. We show here that oxidation of cysteine-106 in DJ-1 is critical for some of these interactions. It is notable that C106 is oxidized at physiological oxidative potentials during periods of oxidative stress (Mitsumoto et al. 2001; Mitsumoto et al. 2001; Kinumi et al. 2004). This is consistent with a model where DJ-1 is basally maintained unbound in an active-ready state. During periods of oxidative or metabolic stress, DJ-1 is modified via C106 oxidation to sulfinic acid, allowing DJ-1 to interact with protein binding partners. These interactions then affect DJ-1's functions, namely promoting cytoprotection and cell survival. Previously, we have shown that DJ-1 acts as a positive regulator of Nrf2 mediated antioxidant transcription (Clements et al. 2006). The results of the present study suggest that DJ-1 acts as a direct sensor or receptor of oxidative stress mediating down stream antioxidant activities.

Ubiquitin has been long associated with neurodegenerative diseases, including Parkinson's disease. Ubiquitin is a prototypic component of protein plaques found in

Parkinson's disease, though the link between Parkinson's disease associated oxidative stress and ubiquitin has not always been clear. A role of DJ-1 in regulating ubiquitin editing functions, such as the effects reported here on the deubiquitinating enzyme Cezanne, suggest a strong association between these two disease processes. It should be noted that another of the handful of genes accounting for monogenetic Parkinson's disease is UCH-L1, a deubiquitinating enzyme (Lincoln et al. 1999; Maraganore et al. 1999; Zhang et al. 2000). Further research is warranted to determine the clinical significance and contribution of deubiquitinating enzyme activity in causing Parkinson's disease.

There is a strong association between Parkinson's disease, ubiquitin biology, and oxidative stress. Similarly, there is a strong association between DJ-1, antioxidant responses, and the regulation of biological pathways in the cell by ubiquitin and ubiquitin-like protein modifications. It has been suggested that DJ-1 disrupts proteosome function, and DJ-1 is known to bind enzymes that mediate small ubiquitin like modifier (SUMO) conjugation. Here we find that DJ-1 binds to Cezanne, an A20 family deubiquitinating enzyme. Additionally, we show that DJ-1 acts in vitro as an inhibitor of deubiquitination. These findings further implicate DJ-1's role in the regulation of ubiquitination as an important mechanism of DJ-1 function.

We find that A20, a ubiquitin editing enzyme that contains deubiquitinating enzyme activity, downregulates Nrf2 mediated responses. Because DJ-1 inhibits deubiquitinating enzyme activity *in vitro* and acts as a positive regulator of Nrf2 activity, it follows that DJ-1 could activate and stabilize Nrf2 by inhibiting A20. However, the effects of DJ-1 on A20 with respect to Nrf2 are modest in our experiments. The goal of

this study is to examine the effects of DJ-1 protein-protein interactions in the context of peripherally derived epithelial cells, like those causing the overwhelming majority of cancers. These cells express high levels of DJ-1. If DJ-1 is not a limiting factor in a pathway, such as the activity of A20 on Nrf2 in these cells, then our over-expression of DJ-1 will fail to see profound effects. However, this does not rule out a role for DJ-1 in regulation of A20.

A20 caused a decrease in Nrf2 protein levels, but this decrease was not accompanied by an increase in Nrf2 ubiquitination over the course of the experiment. Therefore, the mechanism of the Nrf2 effect on A20 remains unclear. In previously published reports, A20 promotes the degradation of RIP1 by catalyzing the deubiquitination of K63 activating ubiquitin from RIP1 allowing K48 ubiquitination to proceed leading to the breakdown of RIP1 (Wertz et al. 2004). If a similar model were to apply to Nrf2, which is continually degraded in the absence of oxidative stress leaving only a very small amount maintained in an active-ready state, then over-expression of A20 could lead to further destabilization of Nrf2 protein prior to oxidative stress. This could account for the lack of Nrf2 protein and Nrf2 ubiquitination seen when A20 is over-expressed.

3.5 MATERIALS AND METHODS:

Cell culture, transfections and luciferase gene reporter assays:

293T, Huh7, and HT-29 cells (ATCC), were maintained in cell culture according to ATCC recommendations. Mouse embryonic fibroblasts (MEFs) were isolated from day 13.5 DJ-1 knockout mouse embryos. MEFs were maintained in Dulbecco's modified eagle medium supplemented with 10% fetal calf serum. All cells were cultured in the presence of penicillin and streptomycin to prevent contamination. Plasmid DNA was transfected into 293T cells using FuGene6 (Roche) while MEFs and Huh7 cells were transfected using Lipofectamine 2000 (Invitrogen), both according to manufacturer instructions.

Plasmid DNA constructs encoding DJ-1 (Hod et al. 1999), A20 (Boone et al. 2004), the NF-κB luciferase reporter construct (Ashburner et al. 2001), the ARE luciferase reporter (Clements et al. 2006), were previously described elsewhere. The C106A DJ-1 mutant was generated by QuickChange mutagenesis. CRE-luciferase plasmid was purchased from Clontech. NadK, BBS1, and Cezanne were all cloned into pcDNA3.1D (Invitrogen) using a directional cloning strategy using the following primer pairs: mNadK: 5'-CACCATGGAAATGGAACAAGAAAAGA-3', 5'-GCTGTCCTCCTCATCCTCTG-3, mBBS1: 5'-ACCCATGGCTGCGGCGTCTTCATC-3', 5'-GGCAGCTGCCAGACCCTCAC-3' and Cezanne/hZa20d1: 5'-CACCATGACCCTGGACATGGATGCTG-3', 5'-GAACCTGTGCACCAGGAGCT-3'. Additionally, Cezanne was also cloned into the

pET101D vector (Invitrogen) for prokaryotic expression using the same cloning strategy and primers.

Luciferase reporter gene assays were performed in 293T cells. Where indicated TNFα treatment was with 20 ng/mL for 6 hours, while tert-butylhydroquinone (tBHQ) (Fluka) was at 5 μM and forskalin (Sigma Aldrich) at 10 μM both for 18 hours. Cells were lysed with Reporter Lysis Buffer (Promega), and analyzed as previously described (Piskurich et al. 1999). Integrated light production was normalized between samples performed in triplicate by protein concentration determined by bicystronic acid protein assay (BCA).

Recombinant Cezanne protein production and purification:

pET101-Cezanne was transformed into chemically competent bacteria from the protease depleted Lac responsive E. Coli strain BL21. Cultures were grown to log phase growth as measured by OD600, and protein expression induced by 1 mM incubation with the sugar analogue isopropyl-β-D-thioglacatopyranoside (IPTG) for 4 hours at 37C. The bacterial cultures were lysed as previously described (Clements, McNally et. al. 2006), and His-tagged Cezanne protein was purified by metal coordination with a nickel-nitrillotriacetic acid (Ni-NTA) (Qiagen), and eluted with imidizole. Protein purity was assessed by SDS-PAGE followed by coomassie R-250 (Biorad) protein staining or western blotting using anti-V5 (Invitrogen) or anti-Cezanne (Aviva Biosystems) antibodies. Generation of recombinant full-length DJ-1 was reported previously (Clements et al. 2006).

Immunoprecipitation and antibody applications:

Rabbit polyclonal anti-DJ-1 antibody generated against full-length recombinant DJ-1 protein was characterized previously. Anti-Flag M2-agarose (Sigma), and anti-V5 agarose (Sigma) were used for immunoprecipitation of epitope tagged proteins. Immunoprecipitation of Flag-DJ-1 and Flag-C106A from MEF cultures for mass spectrometric analysis was done using anti-Flag M2-agarose gel (Sigma), and eluted using synthetic Flag peptide (Sigma). Protein complexes were eluted using a vast molar excess of Flag peptide, column desalted (Amersham), and concentrated in an Ultrafree centrifugal concentrator with a molecular weight cut off of 10000 daltons (Millipore). Trace amounts of Flag peptide remaining were removed by filtration of the protein solution through a ZipTipC18 (Millipore).

Western blot analysis was performed by first SDS/LDS gel electrophoresis through polyacrylimide gels, and then SDS-free electro transfer to nitrocellulose using a semi-dry transfer apparatus (Biorad). Membranes were blocked in 5% nonfat dry milk, and washed using TBS-T (10 mM Tris pH7.6, 150 mM NaCl, 0.05% Tween-20). All primary antibody incubations were performed overnight at 4C. Secondary HRP-conjugated antibodies (Santa Cruz), were incubated for 30 min at room temperature. Immunoreactive bands were detected using chemoluminescent substrate (Pierce) and exposure to X-Ray Film (Kodak).

Nrf2 ubiquitination assays were performed in 293T cells transfected with V5-Nrf2 and HA-Ubiquitin. The cells were lysed in 400 µl SDS lysis buffer (50 mM Tris-HCl pH7.5, 0.5 mM EDTA, 1% SDS, 1 mM DTT) and boiled for 10 minutes. Cellular debris was pelleted and SDS concentrations were diluted by the addition of 1100 µl 0.5% NP-40

lysis buffer with added protease inhibitors. Anti-V5 agarose was then added and incubated for 14-16 hours. The agarose matrix was washed extensively with 0.5% NP-40 lysis buffer and the proteins were eluted by boiling in 2x loading buffer with DTT. The eluates were then analyzed by western blot analysis for the presence of HA-Ubiquitin.

Quadrupole-time of flight (Q-TOF) MS/MS:

Quadrupole time of flight (Q-TOF) mass spectrometric analysis was performed to identify protein components in solution. Protein mixtures were digested by the protease Trypsin in a 50:1 substrate to enzyme ratio, for 4 hours at 37C. The resulting peptide samples were tested to ensure no residual detergent was present, and then run on an electro spray Q-TOF tandem mass spectrometer (MS/MS), preceded by an LC column to remove any trace impurities. Peptide mass signatures were then scored by the confidence values to rank peptide identifications, where a score of greater than 32 was conservatively determined to be positive. Mass spectra were searched across several existing databases using the Mascot search engine (Matrix Science), to identify proteins fitting the peptide spectra. These data were then analyzed further by Y and B ion series sequences to ensure positive identification. For interaction analysis, peptides cooresponding to trypsin or immunoglobins, which were exogenously added to the samples were removed from analysis. Furthermore, in each experiment, samples were run to control for nonspecific binding, and all peptide masses obtained from those samples were subtracted as background. These peptides generally matched those from cytoskeletal contaminants, such as keratin. A list of the peptide masses removed from analysis in this way are containing in supplemental table 3.8.

Deubiquitination and peptidase assays:

In vitro deubiquitinating enzyme assays were carried out as previously described (Evans et al. 2003; Wertz et al. 2004). In brief, the a deubiquitinating enzyme was reacted with a branched ubiquitin substrate containing between 2 and 7 ubiquitin monomers (Biomol), in a buffer containing 50 mM HEPES pH 8.0. 0.01% Brij-35, 3 mM DTT. Reactions were carried out at 37C for 4 hours, and assessed by western blot analysis for ubiquitin. Ubiquitin oligomers could be differentiated by molecular weight, allowing an assessment of cleavage.

An AMC peptide mimicking the carboxy terminal sequence of ubiquitin, Z-Arg-Leu-Arg-Gly-Gly-AMC (Bachem), was incubated with Isopeptidase-T at room temperature for up to 5 hours in a microtiter plate, and fluorescence intensity was measured at various time points using a microplate spectrofluorimeter, where samples were excited by 360 nm light, and emission read at 460 nm.

Figure 3.1: Mass spectrometric identification of DJ-1 interacting proteins (**A**) Table summarizing proteins identified by peptide mass signature and sequencing isolated in DJ-1 containing protein complexes. In the absence of oxidative treatment with hydrogen peroxide, no proteins other than DJ-1 were identified in complex with DJ-1. Four novel DJ-1 interacting proteins were identified in DJ-1 complexes following hydrogen peroxide treatment, but none of these proteins were found in complex with C106A mutant DJ-1. These results show that DJ-1 protein-protein interactions require cysteine-106 oxidation. (**B**) DJ-1 co-immunoprecipitates with Cezanne and BBS1. Immunoprecipitation of either V5-Cezanne or V5-BBS1 pulls down co-expressed Flag-DJ-1. In the absence of V5-tagged protein expression or in the presence of the unrelated protein control V5-WDR5, Flag-DJ-1 is not immunoprecipitated by the anti-V5 antibody. Duplicate lanes representing hydrogen peroxide treatment are shown for each protein.

Figure 3.1

A.

pcDNA	DJ-1	C106A	DJ-1 +H2O2	C106A +H2O2
	DJ-1	DJ-1	DJ-1	DJ-1
			BBS1	
			Cezanne	
			OATP-r	
			NadK	

B.

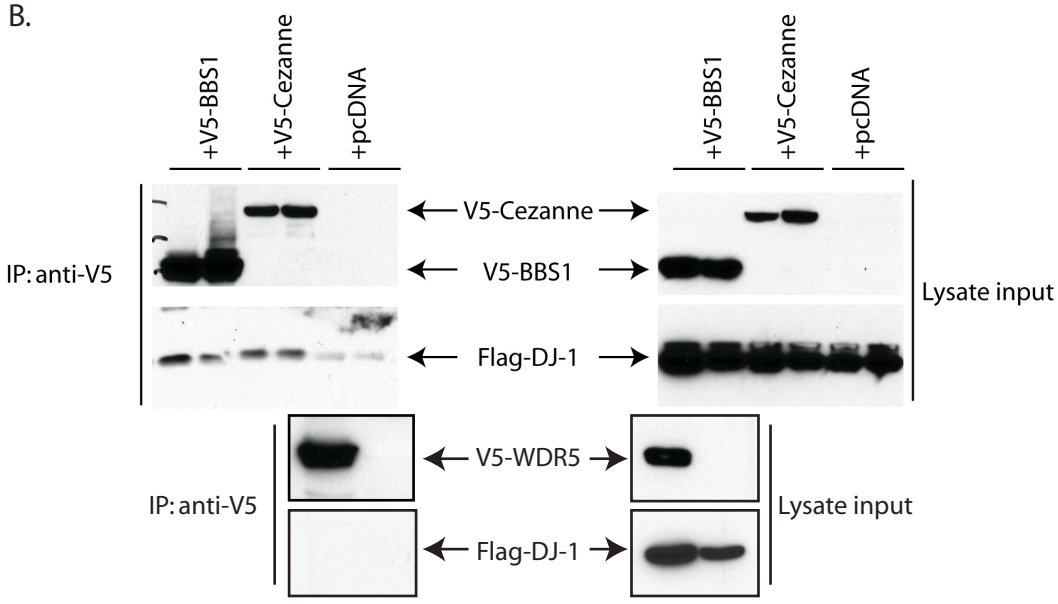


Figure 3.2: Gel filtration chromatography of DJ-1 protein complexes Using a SuperDex-75 gel filtration column to separate molecules by molecular size, we separated (**A**) purified bovine serum albumin (molecular weight 66 kDaltons), (**B**) recombinant DJ-1 (molecular weight 24 kDaltons as a monomer) and (**C**) whole cell lysate from HT-29 colon carcinoma cells. The SuperDex-75 column excludes complexes over 100 kDaltons molecular weight, which migrates in the void volume in fraction 8-9. Smaller molecular weight complexes can enter the matrix are separated by size, with small molecules being retained on the column longer. Western blot analysis of DJ-1 from the HT-29 fractions shows that all of the DJ-1 protein was eluted in the same fractions (19-22) as recombinant DJ-1, suggesting that DJ-1 exists basally as a monomer or homodimer in these cells.

Figure 3.2

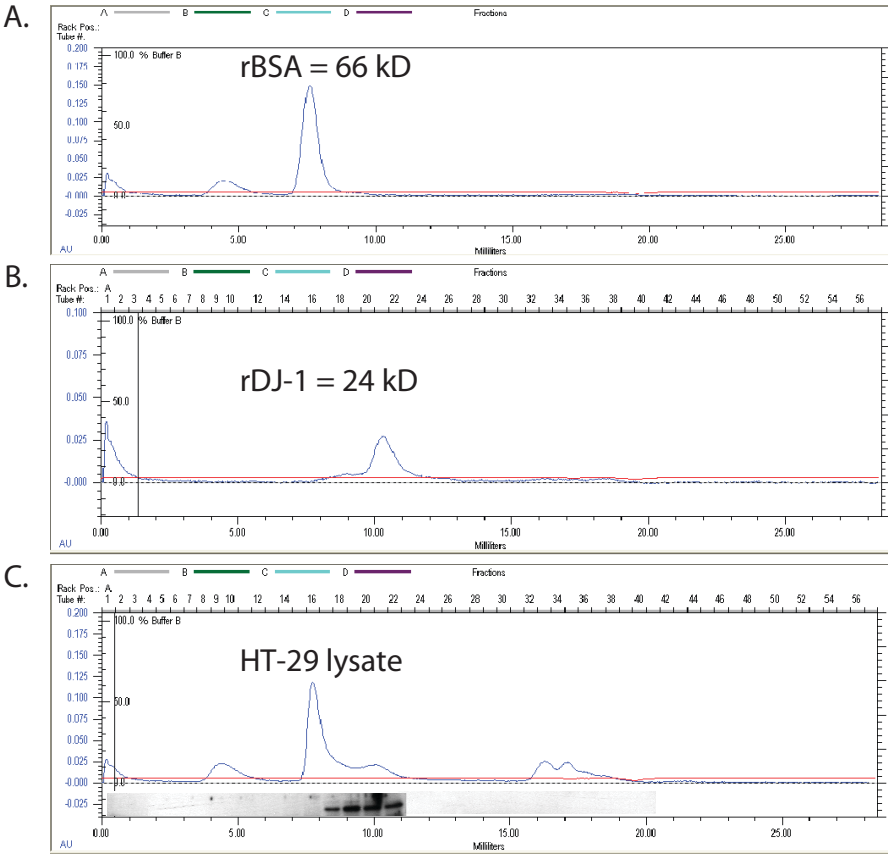

A.
rBSA = 66 kD
B.
rDJ-1 = 24 kD
C.
HT-29 lysate
Fractions
Milliliters
AU

Figure 3.3: Cellular localization of putative DJ-1 interacting proteins identified from mouse embryonic fibroblast cells. Confocal microscopy was used to image Huh7 liver hepatoma cells expressing epitope tagged proteins stained by antibodies against (**A**) Flag-DJ-1 (**B**) V5-BBS1 (**C**) V5-NadK or (**D**) V5-Cezanne. Immunolocalization shows that these proteins share a cytoplasmic staining pattern with DJ-1.

Figure 3.3

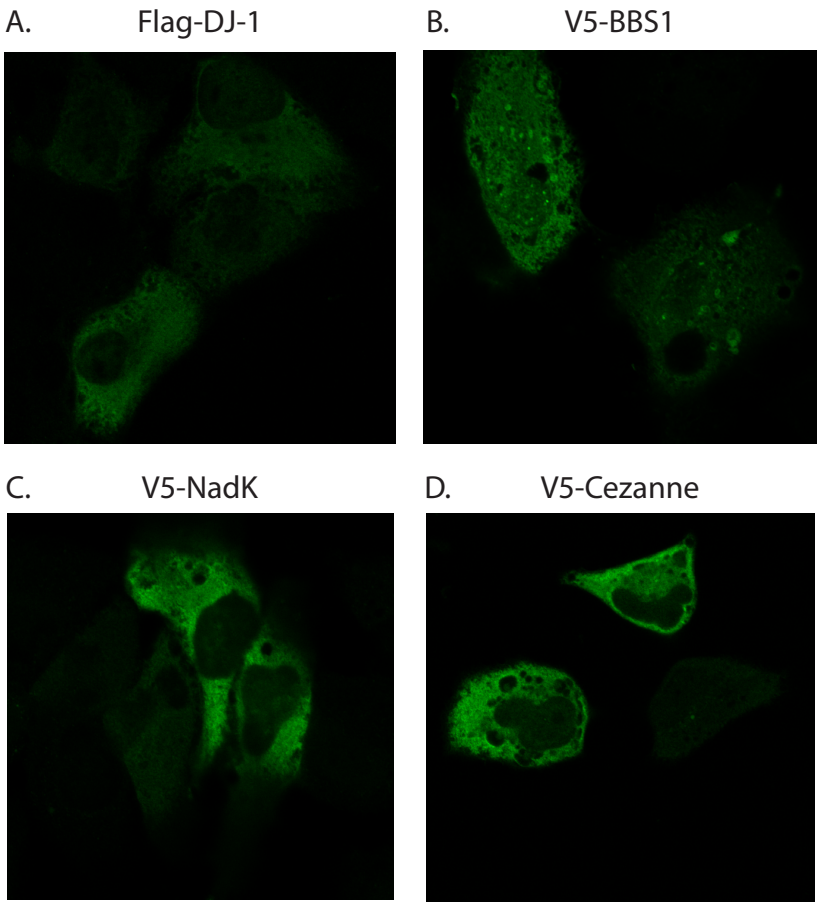
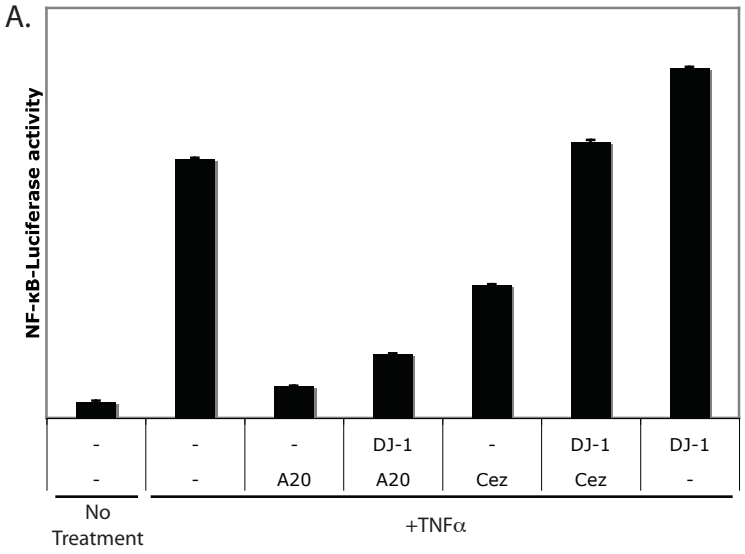


Figure 3.4: DJ-1 inhibits Cezanne mediated anti-NF-κB activity 293T cells containing luciferase reporter constructs were transfected with the deubiquitinating enzymes A20, or Cezanne in the presence or absence of over-expressed DJ-1 (**A**) Luciferase under control of an NF-κB responsive promoter is activated by treatment with TNFα. Transfection with either A20 or Cezanne down regulates NF-κB transcription leading to lower luciferase activity. Co-expression of DJ-1 moderately affects the inhibition of NF-κB by A20 but completely reverses the effect Cezanne restoring NF-κB transcription to normal levels. (**B**) cAMP-responsive element controlled luciferase expression is unaffected by transfection with A20, Cezanne, or DJ-1 showing the effect of the deubiquitinating enzymes is a specific effect and not a global inhibition of signaling pathways or transcription.

Figure 3.4


A.
NF-κB-Luciferase activity
- - - DJ-1 - DJ-1 DJ-1
- - A20 A20 Cez Cez -
No Treatment
+TNFα

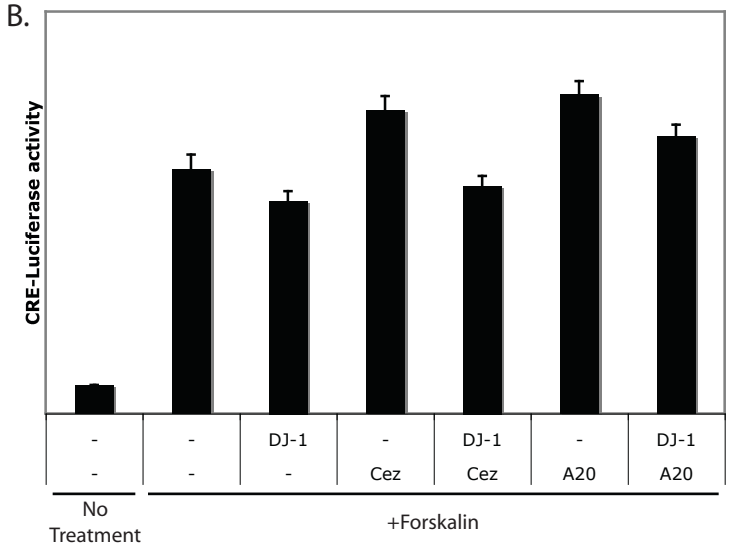

B.
CRE-Luciferase activity
- - DJ-1 - DJ-1 - DJ-1
- - - Cez Cez A20 A20
No Treatment
+Forskalin

Figure 3.5: DJ-1 counteracts the function of deubiquitinating enzymes Recombinant Cezanne (rCez) was expressed in E.Coli, purified over a Ni-NTA column, and eluted by increasing concentrations of imidizole. Fractions were collected and analyzed for the presence of Cezanne. These fractions were analyzed by (**A**) coomassie stain, (**B**) anti-V5 epitope immunoblotting, and (**C**) anti-Cezanne immunoblotting. The fractions contained a mixture of full-length Cezanne and several degradation products. All bands on the coomassie stained gel were reactive with either the anti-V5 antibody or the anti-Cezanne antibody suggesting a pure sample. (**D-E**) *In vitro* deubiquitinating enzyme assay in which recombinant ubiquitin chains linked by either (**D**) K48 or (**E**) K63 bonds were reacted with recombinant Cezanne in the absence of DJ-1 or with increasing levels of rDJ-1 titrated from a molar ratio of 1:1 to 1:8 , Isopeptidase-T is used as a control since it breaks down branched ubiquitin to a monomer. DJ-1 prevented Cezanne mediated deubiquitinating enzyme activity showing a dose dependent inhibition. (**F**) Using a peptide mimicking the carboxy terminus of ubiquitin linked to the flurochrome, AMC, we recorded the activity of the deubiquitinating enzyme, Isopeptidase-T. While Isopeptidase-T was able to break down the peptide increasing fluorescence, the addition of rDJ-1 inhibited the activity of Isopeptidase-T (IsoT) in a dose dependant manner showing greater than 50% inhibition at a 4:1 molecular ratio of DJ-1 to Isopeptidase-T.

Figure 3.5

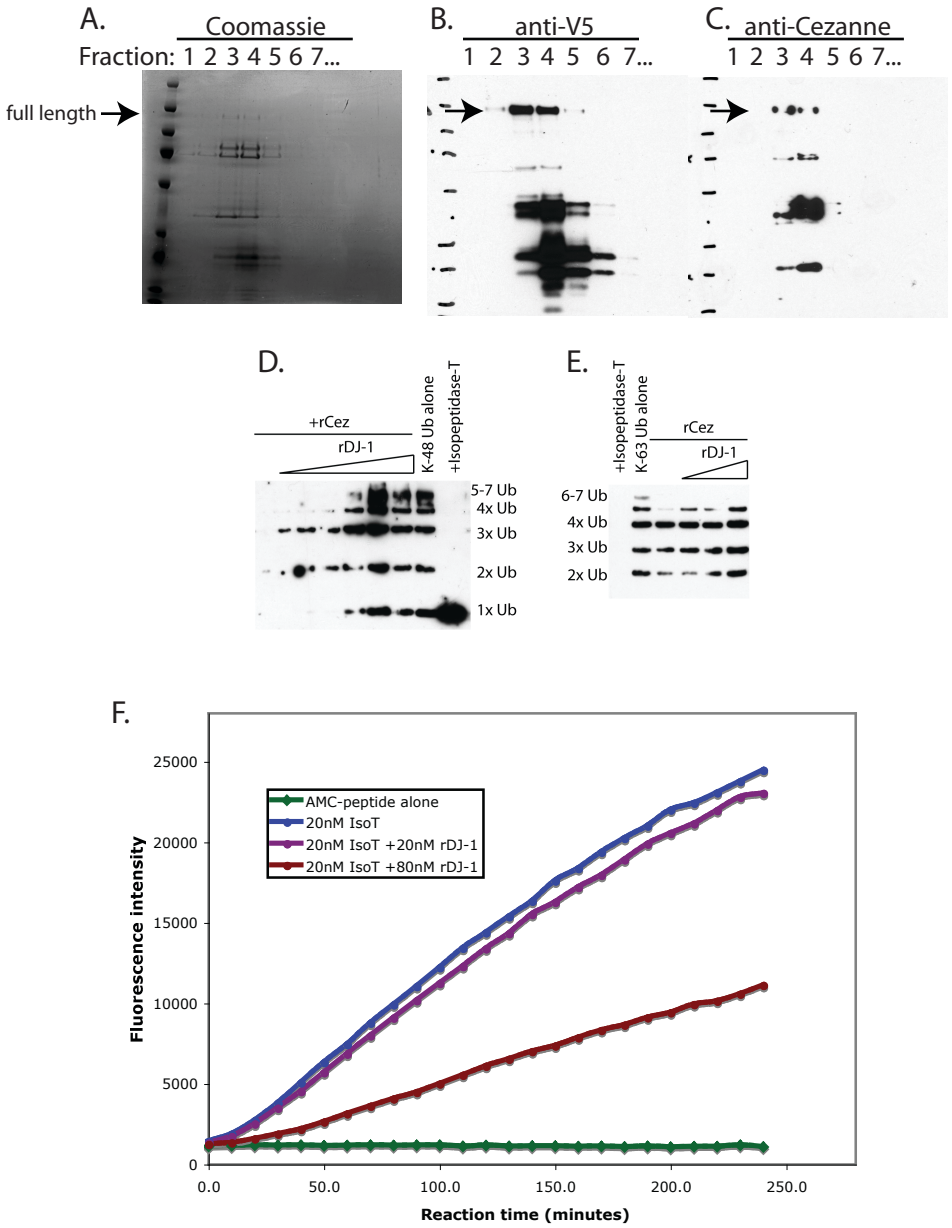

A.
Coomassie
Fraction: 1 2 3 4 5 6 7...
full length
B.
anti-V5
1 2 3 4 5 6 7...
C.
anti-Cezanne
1 2 3 4 5 6 7...
D.
+rCez
rDJ-1
K-48 Ub alone
+Isopeptidase-T
5-7 Ub
4x Ub
3x Ub
2x Ub
1x Ub
E.
+Isopeptidase-T
K-63 Ub alone
rCez
rDJ-1
6-7 Ub
4x Ub
3x Ub
2x Ub
F.
AMC-peptide alone
20nM IsoT
20nM IsoT +20nM rDJ-1
20nM IsoT +80nM rDJ-1
Fluorescence intensity
25000
20000
15000
10000
5000
0
0.0
50.0
100.0
150.0
200.0
250.0
Reaction time (minutes)

Figure 3.6: A20 downregulates Nrf2 mediated transcription. Using a luciferase reporter gene construct under control of an antioxidant response element (ARE) that is regulated by Nrf2 activity, the effects of deubiquitinating enzyme activity was assessed with respect to Nrf2. DJ-1, a positive regulator of Nrf2, when over-expressed modestly increases Nrf2 transcriptional activity in these cells containing abundant endogenous DJ-1. Expression of Cezanne has no effect on Nrf2 mediated transcription in this experiment, but the related ubiquitin editing enzyme A20 causes a profound loss of Nrf2 mediated transcription. Co-expression of DJ-1 is unable to rescue this phenotype.

Figure 3.6

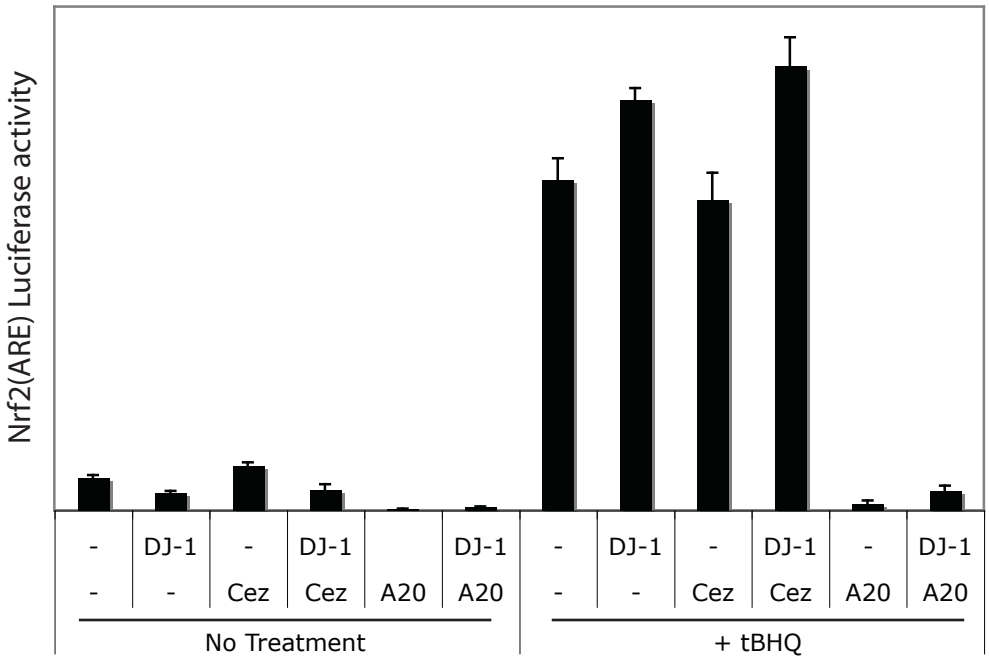

Nrf2(ARE) Luciferase activity
- DJ-1 - DJ-1 DJ-1 - DJ-1 - DJ-1 - DJ-1
- - Cez Cez A20 A20 - - Cez Cez A20 A20
No Treatment
+ tBHQ

Figure 3.7: A20 reduces Nrf2 protein expression independent of Nrf2 ubiquitination. (**A**) Protein lysates from 293T cells transfected with A20 or pcDNA in the presence or absence of over-expressed DJ-1 and tBHQ were blotted for Nrf2 protein expression or G3PDH as a loading control. Expression of A20 causes a decrease in Nrf2 protein levels detected following tBHQ induction, and this reduction is relatively unaffected by DJ-1 co-expression. (**B**) A ubiquitination assay of examining the levels of ubiquitinated Nrf2 accumulation in cells over the course of a 5 hour treatment with the proteosome inhibitor, MG132. V5 epitope tagged Nrf2 was expressed in 293T cells with varying expression of A20 and DJ-1. Denatured Nrf2 was then immunoprecipitated and blotted for the presence of ubiquitin moieties. In the absence of DJ-1 and A20 over-expression, Nrf2 is ubiquitinated. The addition of DJ-1 decreases the ubiquitination of Nrf2, while the addition of A20 reduces ubiquitinated Nrf2 to undetectable levels. The co-expression of A20 and DJ-1 remains at undetectable levels. Controls are included without the expression of V5-Nrf2 that show the ubiquitination observed in the left panel is specific.

Figure 3.7

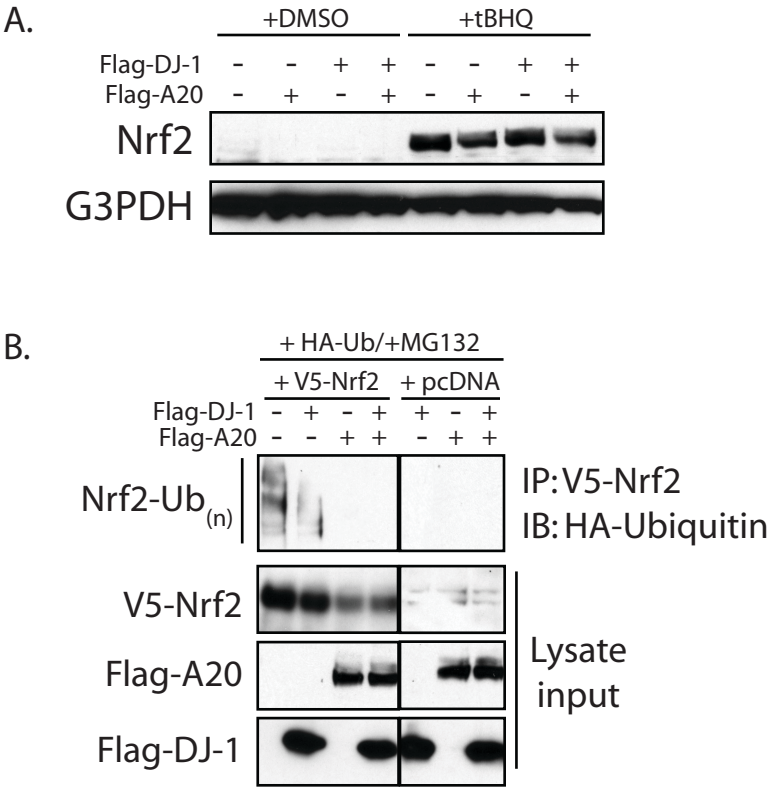

A.
+DMSO
+tBHQ
Flag-DJ-1 - - + + - - + +
Flag-A20 - + - + - + - +
Nrf2
G3PDH
B.
+ HA-Ub/+MG132
+ V5-Nrf2
+ pcDNA
Flag-DJ-1 - + - + + - +
Flag-A20 - - + + - + +
Nrf2-Ub(n)
IP: V5-Nrf2
IB: HA-Ubiquitin
V5-Nrf2
Flag-A20
Flag-DJ-1
Lysate input

Table 3.8: Peptide masses excluded from analysis in quadrupole mass spectrometry (Q-TOF). This table lists the peptide masses found in the negative control immunoprecipitations (pcDNA anti-Flag). Since these controls did not immunoprecipitate any specific expressed protein in the lysates, these peptides represent non-specific contaminants in the solutions, and were therefore removed from analyses.

Table 3.8

Background peptide masses removed from anaysis:

Trypsin	Albumin:	Keratin1	Keratin9	Keratin-other	Keratin-orthologs
421.7	464.23	487.25	530.77	602.31	516.3
523.28	507.8	590.3	579.28	655.36	545.76
710.85	547.3	633.3	793.9	665.36	555.24
737.68	582.31	650.77	837.39	730.91	583.28
1106.05	653.36	651.86	968.15	752.69	601.3
1142.08	740.4	679.365	1088.86		683.31
	756.43	686.36			691.32
	769.4	692.35			695.84
	784.35	738.4			854.39
	820.46	858.93			998.99
	978.49				

CHAPTER IV:

CONCLUSIONS

CONCLUSIONS

At the time this thesis work was initiated, DJ-1 was a gene product of unknown significance. There were a total of nine publications regarding DJ-1 that ascribed various functions to the protein without significant mechanistic evidence. We identified DJ-1 in a whole proteome screen of proteins altered following combination chemotherapy in non-small cell lung carcinoma cells (NSCLC) (MacKeigan et al. 2003). The protein spot representing DJ-1 on 2-dimensional electrophoresis was significantly diminished in cells treated with a combination chemotherapy that would ultimately lead to enhanced cell death. We did not know the functional significance of DJ-1 protein in this system, so we restored DJ-1 expression to the NSCLC cells and found that cells expressing higher levels of DJ-1 were protected from cytotoxic cell killing. To that point, DJ-1 had been associated with effects on gene expression and transformation, but not on response to toxic cell death. The work of this thesis work describes (1) a mechanism by which DJ-1 acts to protect cells from cytotoxic insults, and perhaps more importantly (2) puts several other investigators' research into perspective with respect to DJ-1 and human disease states – namely cancer and Parkinson's disease.

The effects of a single gene or protein on the biology of an organism are of debatable importance and DJ-1 is no different. Generally, genes that regulate the function of a large number of other biological pathways are of central biological importance. For example, the tumor suppressor p53, which helps regulate the coordinated process of apoptotic cell death. Similar to p53, its association with disease states underscores DJ-1's significance. While the actual causes of Parkinson's disease

have remained elusive, DJ-1 is one of only a very small handful of genes that when altered cause Parkinson's disease in people without any other identified defect (Bonifati et al. 2003). Predating the association of DJ-1 with Parkinson's disease is its effects on cell survival. In fact, DJ-1 was first identified for its ability to transform cells in culture to a more cancer-like phenotype (Nagakubo et al. 1997). To date, DJ-1 is the only known gene that is directly associated with both Parkinson's disease and cancer. Given that Parkinson's disease is caused by the loss of DJ-1, while cancerous states involve increased DJ-1 expression – it stands to reason that Parkinson's disease and cancer lie on opposite ends of a spectrum determined by DJ-1's function: namely, cell death. The thesis work concluded here also suggests that this cell death affected by DJ-1 is toxic cell death accompanied by oxidative stress.

Given DJ-1's strong disease association, determining the function of DJ-1 and its molecular mechanisms are important for two related but distinct reasons. First, since DJ-1 is a cause of Parkinson's disease, insights into the function of DJ-1 (which when lost cause Parkinson's disease) could offer important insight into the causes of Parkinson's disease in general. Parkinson's disease has been extensively studied, yet at its earliest stages is still poorly understood. Any hope for targeted curative or preventative approaches to Parkinson's disease absolutely require an understanding of the cause of disease. Monogenetic subtypes, like DJ-1 mutation, are widely thought to offer the opportunity to understand mechanisms underlying Parkinson's disease. On the other hand, the mechanisms underlying the causes of cancer are becoming clearer with recent understanding of oncogene/tumor suppressor biology. While DJ-1 is an oncogene, having profound effects on transformation in culture and association with tumors in

patients, DJ-1 does not have the classical functions of an oncogene. DJ-1 does not affect cell cycle progression or allow escape from checkpoints within a cell's biology. While DJ-1 does prevent cell death, this effect is only apparent following toxic insult, a clear difference from other defined oncogenes. This seemingly subtle difference may underscore a completely new mechanism of cellular transformation, and underlines the importance of responses to environmental factors in governing both tumorigenesis and tumor survival – as well as define the link between cancer and Parkinson's disease we see with DJ-1.

The second reason that understanding DJ-1 function may be important to biomedical science also relies on the causative relationship of DJ-1 with cancer and Parkinson's disease. Namely, that DJ-1 offers a novel target of therapy. The development of the kinase inhibitor Gleevec (formerly STI571), underscores the importance of targeting causal mechanisms in cancer. The thinking goes that, targeting the primary deficiency or malfunction causing a cancer produces anti-tumor effects that, unlike secondary changes in a cancer's cell biology, cannot be circumvented by the cancer. In other words, the primary causes of cancer do not have the level of redundancy that secondary changes do. Future studies will be required to see if inhibition or ablation of DJ-1 function can in fact effectively treat cancers in patients. Likewise, it is as yet unclear that adding DJ-1 function back to either DJ-1 deficient Parkinson's disease patients, or idiopathic Parkinson's disease in general, will be able to reverse or protect from Parkinson's disease.

In chapter 2 of this thesis, we present the first association of DJ-1 with the master regulator of antioxidant transcription, Nrf2. Although other research has shown that DJ-1

is able to protect cells from oxidative and toxic insults, no mechanism had previously been able to account for the profound effect of DJ-1 in this respect. However it was shown that the free radical scavenging ability of DJ-1, through its direct oxidation (i.e. C106 etc), was not able to account for these effects (Takahashi-Niki et al. 2004). We show herein that loss of DJ-1 in NSCLC cells led to the profound loss of antioxidant enzymes. Of particular interested is the significantly decreased expression of NAD(P)H Quinone Oxidoredutase-1 (NQO), which is a prototypic target of Nrf2. NQO1 function is thought to be regulated entirely by its expression, and its expression is dependent on gene transcription, which is governed by Nrf2 (Jaiswal 2000). Therefore, the decreased NQO1 mRNA expression in DJ-1 knockdown cells strongly implicated a role of DJ-1 in Nrf2 mediated transcription. Indeed, examination of the promoters of genes identified along with NQO1, shows a strong association with putative Nrf2 binding sites in nearly half of the genes identified.

The mechanism by which DJ-1 affects Nrf2 activity is consistent with the generally accepted mechanism of regulated Nrf2 responses. Namely, Nrf2 is regulated by stability of the Nrf2 protein, not by its own transcription. In chapter 2 we show that DJ-1 functions to stabilize Nrf2 protein, allowing its activation. DJ-1 does this by allowing Nrf2 to dissociate from its cytosolic inhibitor protein Keap1, where Nrf2 can then translocate to the nucleus and activate responsive genes. In the absence of DJ-1, as in the case of siRNA knockdown or genetic ablation of DJ-1, Nrf2 remains bound to Keap1, which targets Nrf2 for ubiquitination and subsequent degradation. The absence of DJ-1 has a striking effect on Nrf2, causing almost complete loss of Nrf2 protein. Therefore, in cells lacking DJ-1 expression, Nrf2 is not active basally, and can no longer

be induced by toxic stimuli or mimetics. This suggests a model of DJ-1/Nrf2 axis, where DJ-1 maintains an active-ready pool of Nrf2 protein in the absence of toxic stresses, which can then be activated during periods of stress to protect the cell. The loss of DJ-1 leads to Nrf2 degradation, and affected cells can no longer respond to toxic stresses – leading to a lack of cytoprotection during environmental insults. We also showed that this loss of DJ-1 affected several endogenous Nrf2 regulated genes, and that these effects were conserved between the human tumor cell lines and the primary mouse cell cultures suggesting that this is an important function of DJ-1 for the organism.

While we have established a strong functional link between DJ-1 and Nrf2 in chapter 2, it remained unclear how DJ-1 physically exerted its effects. We hypothesized that DJ-1 effects were mediated through protein-protein interactions since no enzymatic activities of DJ-1 have been identified, and oxidative modification of DJ-1 alters its binding surface surfaces (Lee et al. 2003). Yet, we were unable to find DJ-1 in physical association with several Nrf2 pathway mediators including Nrf2 itself, the inhibitor protein Keap1, or the ubiquitin targeting machinery including Cullin-3. Therefore, we adapted unbiased approaches to survey the interactions of DJ-1 in chapter 3 to determine the important mechanisms of Nrf2 mediated cytoprotection, but also other general effects of DJ-1 with respect to cellular biology.

Both direct and indirect DJ-1 protein interactions in the cell were identified by isolating DJ-1 containing protein complexes and then using in solution mass spectrometry to identify proteins by peptide mass signatures. To our surprise, DJ-1 in these cells was not bound to other proteins but instead remained only as a monomer/self-associating dimer. However, when the cells were treated with an oxidant, in this case

hydrogen peroxide, DJ-1 formed higher order complexes with other proteins. The most susceptible residue to oxidation at physiologic oxidative potentials is C106 (Kinumi et al. 2004). This cysteine is maintained on the surface of DJ-1 with bond angles that are strained to allow for exposure, is highly conserved among DJ-1 orthologs (Honbou et al. 2003; Huai et al. 2003; Lee et al. 2003; Tao et al. 2003; Wilson et al. 2003), and previous studies have shown that this residue is necessary for DJ-1 functions (Takahashi-Niki et al. 2004; Zhou et al. 2006). However, since DJ-1 forms homodimers in cells, and since the exact role of C106 in dimer formation is unclear, we could not simply express epitope tagged C106 mutant DJ-1 in cells that expressed wild type DJ-1 because isolated complexes could contain wild type DJ-1 protein complexes. Therefore, we expressed mutated C106A DJ-1 protein in mouse embryonic fibroblasts (MEFs) generated from DJ-1 knockout mice. In this way we could compare wild type and C106A protein complexes with respect to cysteine 106 oxidation. We found that DJ-1 did not form complexes with other proteins in the absence of oxidative stress in either case. Furthermore, when oxidized protein associations found in complex with wild type DJ-1 were not present in C106A mutants. Indeed, we could identify no proteins bound to C106A mutant DJ-1 at all. This is consistent with a model where DJ-1 remains unbound in the absence of oxidative stress, but in the presence of stressors cysteine 106 becomes oxidized leading to function DJ-1 complex formation.

We identified several novel DJ-1 interacting proteins in both transformed human and primary mouse cells, and have gone on to functionally characterize one such interaction, hZa20d1 also known as Cezanne. We chose this protein because of its role on ubiquitination, which could parallel Nrf2 regulation, and because of the strong disease

association between ubiquitin/proteosome activity and Parkinson's disease and cancer. While the clinical significance of Cezanne in these diseases is unknown, Cezanne does negatively regulate NF-κB transcription – NF-κB activity is associated with tumor survival (Liu et al. 1996; Wang et al. 1996). Therefore, we hypothesized that since DJ-1 is transforming, it would function to up-regulate NF-κB activity, and therefore would negatively regulate Cezanne. Using NF-κB Luciferase gene reporter assays we showed that this was in fact the case. Not only did DJ-1 modestly upregulate NF-κB activity, but it completely abrogated the negative effect of Cezanne. We showed that this effect on Cezanne occurred in vitro with only DJ-1 and Cezanne present using recombinant deubiquitinating enzyme assays, suggesting that DJ-1 affects Cezanne activity directly. Furthermore, we demonstrate that DJ-1 is able to inhibit even the strong deubiquitinating enzyme, Isopeptidase-T in ubiquitin mimicking peptidase assays. These experiments suggest a broader role for DJ-1 in regulating deubiquitinating enzymes. The significance of this effect on Nrf2 and more broadly on ubiquitin mediated signaling in the cell is a subject of ongoing research.

Our analyses regarding DJ-1 function suggest a model of DJ-1 as a receptor for oxidative stress. In brief, DJ-1 remains unbound in cells in the absence of stress. This pool of unbound DJ-1 acts as an active-ready pool of oxidative sensors. When cells undergo oxidative stress from external toxin exposure, immune activation, or unhindered oxidative metabolism, the cysteine at residue 106 in DJ-1 is oxidized to a sulfinic acid invoking a conformational change in DJ-1 protein. This oxidative modification allows DJ-1 protein to form higher order protein complexes including but not limited to ubiquitin

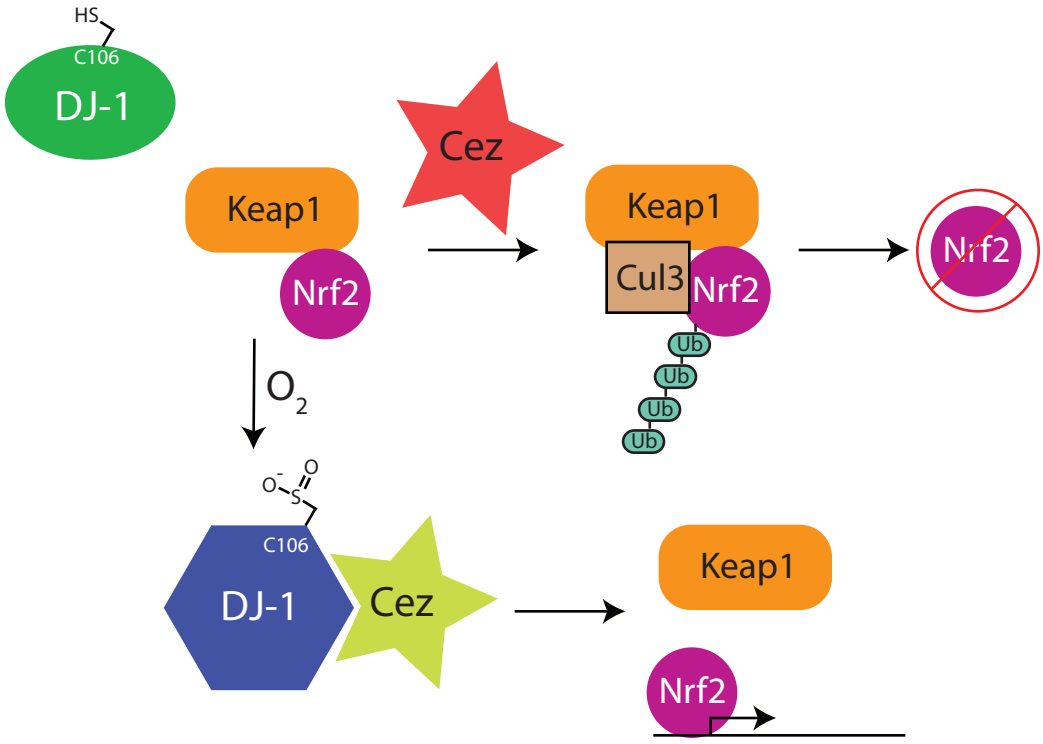
editing proteins such as Cezanne or A20. (Figure 4.1) These enzymes and others then exert DJ-1's antioxidant effect on cell signaling pathways activating antioxidant response pathways, such as Nrf2 and its regulated genes including NQO1. The sum effect is increased cellular protection and survival. Deregulation of this control axis leads to disease states. In the case of over-active or over expressed DJ-1, inappropriate and overzealous protection occurs allowing cells to survive toxic insults generating cancerous cells. Conversely, when DJ-1 activity or expression is lost, cells can no longer survive everyday exposures. In this case, the cells that are most sensitive to oxidative stress would be differentially effected, such as the neurons, leading to cell death which manifests itself clinically as a degenerative defect, namely Parkinson's disease.

The work summarized here represents the first reports of mechanistic evidence on how DJ-1 exerts cytoprotection during periods of toxic and oxidative stress. We have implicated, for the first time, Nrf2 mediated gene transcription and subsequent cytoprotection as a potential mechanism of both cell transformation and tumor progression associated with DJ-1. These results also call into question the role of Nrf2 in Parkinson's disease, which has been a complicated association in the past. En total these results place DJ-1 soundly at the biological border of genetics and environmental exposure.

Figure 4.1 Model of DJ-1 oxidation regulating protein binding In the absence of oxidative stress DJ-1 remains unbound in the cytosol of cells. The Nrf2 antioxidant transcription factor is constitutively degraded leading to down-regulation of antioxidant trascription. We have shown that deubiquitinating enzymes including A20 and possibly its related enzyme Cezanne cause loss of Nrf2 protein.

During periods of oxidative stress the cysteine-106 of DJ-1 is oxidized to a sulfinic acid affecting a change in DJ-1 allowing binding to other interacting proteins, such as Cezanne. In the case of Cezanne, DJ-1 binding inhibits Cezanne activity producing downstream effects such as the stabilization of Nrf2 protein or activation of NF-κB. Therefore, DJ-1 acts as a sensor of oxidative stress leading to the induction of antioxidant signaling, including Nrf2, perhaps by its effects on inhibiting ubiquitin editing enzymes such as Cezanne.

Figure 4.1


HS
C106
DJ-1
Cez
Keap1
Nrf2
Keap1
Cul3
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Nrf2
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Keap1
Nrf2

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